UNIVERSITY OF ALBERTA

Efficacy of Milk and Probiotic LAB Derived Components as Potential Functional Food and Nutraceutical Ingredients

by

Sylvia Kar Wen Chan Remillard ©

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DEDICATION

To Dr. Lech Ozimek, my supervisor, mentor and friend. Thank you for the opportunities and all the life lessons you have taught me. You encouraged me to see beyond my obstacles and fears when I doubted myself and my abilities.

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To my parents Mike and Sue Chan, without your love, dedication and support I could not achieve what I have achieved nor would I have the courage to aim for what I have not yet endeavored to achieve.

This thesis is dedicated in the loving memory of my
Uncle Reverend Paul Chi Man Chan, my wonderful friend Alison Look
and my father in law, Leo Remillard.

Their memories will be cherished and they will truly be missed.

I long to accomplish a great and noble task, but it is my chief duty to accomplish humble tasks as though they were great and noble. The world is moved along, not only by the mighty shoves of its heroes, but also by the aggregate of the tiny pushes of each honest worker.

Helen Keller

Abstract

Functional foods have not only been a phenomenon of the past few decades; 2500 years ago Hippocrates suggested, "let food by thy medicine and medicine be thy food". The Health Canada working definition for functional foods states that a functional food is similar in appearance to, or may be a conventional food, is consumed as part of a usual diet and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions. Agriculture and Agri-food Canada indicate a tremendous potential for developing function foods from dairy.

There is increasing evidence indicating that lactic acid bacteria (LAB) and milk proteins (MP) may modulate many disease states. The majority of studies use LAB of human origin; while dairy LAB represent a large population of bacteria with unexploited potential. Furthermore, studies are beginning to demonstrate that biotransformation of bacterial growth medium results in the liberation of many bioactive proteins/peptides that may also be used to modulate disease. There is a strong lack of understanding regarding the mechanism through which these compounds alter disease state; in addition, there is a strong need to have well characterized functions of individual ingredients before introduction as a functional food ingredient.

Thus, the objective of this thesis research was to identify new functional ingredients derived from dairy LAB isolates and proteins that can be used as dietary adjuvants in the modulation of gastrointestinal disorders. The following biomarkers were investigated: antimutation, adhesion properties and mechanisms of adhesion of LAB, apoptotic, necrotic and cytotoxic action of LAB and MP against cancer cells. We also investigated the ability of LAB isolates and MP to modulate the secretion of TNFα, IL-6 and nitric oxide from macrophage cells. We found that each of the LAB tested was strongly adhesive and the adhesion mechanisms varied in a strain dependent manner. We found that LAB and MP were able to prevent mutation, induce cellular death and modulate the immune response, in a strain or MP specific manner. These findings and their possible applications in gastrointestinal disease modulation will be discussed.

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LIST OF ABBREVIATIONS

Abbreviation	**Term**
2 AF	2-Aminofluorene
24 H SN	24 Hour Fermented Milk Supernatant
48 H SN	48 Hour Fermented Milk Supernatant
AMRS	Acidified MRS Broth
AOM	Azoxymethane
BCG	Bacillus Calmette Guerin
CD	Crohn's Disease
CF	Cystic Fibrosis
DMH	Dimethylhydrazine
DMSO	Dimethylsulfoxide
FCS	Fetal Calf Serum
Glu-P-1	2-amino-6-methyldipyridol[1,2-a:3'2'-d]imidazole
IBD	Inflammatory Bowel Disease
IL 6	Interleukin 6
iNOS	Inducible Nitric oxide synthas
LAB	Lactic Acid Bacteria
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NF	2-Nitrofluorene
NO	Nitric Oxide
NPD	4-nitro-*O*-phenylenediamine
NQNO	4-nitroquinoline-*N*-oxide
PBS	Phosphate Buffered Saline
PG	Peptidoglycan
SEM	Scanning Electron Microscopy
TLR	Toll like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumour Necrosis Factor
Trp-P-1	3-amino-1,4-dimethyl-*5H*-pyrido[4,3-*b*]indole
Trp-P-2	3-amino-1-methyl-*5H*-pyrido[4,3-*b*]indole
UC	Ulcerative Colitis

CHAPTER 1[*]

Introduction

1.1 INTRODUCTION

Although functional foods seem to be a phenomenon of the last two decades, the concept of functional foods is not new. Over 2500 years ago Hippocrates suggested, "let food be thy medicine and medicine by thy food". It has only been since the early 1980's that the Japanese started to use the term functional foods to describe foods that encompass potentially bioactive components that can potentially modulate health beyond inherent basic nutrition. The Institute of Medicine of the US National Academy of Sciences defines functional foods as 'those foods in which the concentration of one or more ingredients have been manipulated or modified to enhance their contribution to a healthful diet (Committee on Opportunities in the Nutrition and Food Sciences, 1994). Recently modifications to this definition have been proposed. Contor (2001) suggested that functional foods should be defined as 'foods that can be satisfactorily demonstrated to affect beneficially on one or more target functions in the body, beyond adequate nutritional effects, in a way relevant to an improved state of health and wellbeing and/or reduction of risk of disease.'

Despite the lack of consensus on the definition of functional foods, the concept of functional foods has gained much importance both to the food industry sector and to consumers. The industrial sector has responded to the increasing demand of consumers for foods that are flavourful, convenient and attractive, yet healthy by developing functional foods. Through the manipulation of food ingredients, identification and isolation of health modulating components naturally present in food, the industrial sector is attempting to meet this demand.

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[*] A portion of this chapter has been published. Chan, S.K.W., and Ozimek, L. The role of probiotics in antimutation and anticarcinogenesis. In: *Food Science and Product Technology.* Research Signpost. India. 2002, pp. 141-153.

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Functional foods are a relatively new concept within the North American Markets. However, with the Japanese, European and Australian markets, functional foods are much better known estimating to be worth over $50 billion in Europe. In Japan, the establishment of Foods for Specified Health Use (FOSHU) has lead to the development of many functional food products with proven efficacy (Stanton et al., 2005).

Dairy products, in particular fermented dairy products, represent a major share of the functional food market. Epidemiological studies demonstrate that consumption of yogurt can decrease the incidence of cancer (Van't Veer et al., 1989; Kojima et al., 2004). In addition, *in vivo* experiments demonstrate the efficacy of yogurt in reducing diarrhea (Isolauri et a., 1994), alleviation of lactose intolerance symptoms (de Vrese et al., 2001), and antimicrobial effects (Kabir et al., 1997).

Fermented dairy products are traditionally produced through the use of lactic acid bacteria (LAB). LAB, as the name implies, through a series of bacteria associated enzymes uses milk as a substrate and produces lactic acid as a major metabolite during growth. Through enzymatic or lactic acid denaturation and/or proteolysis of the native milk protein LAB liberate bioactive peptides and proteins. These are referred to as biogenics (Stanton et al., 2005).

The body of scientific evidence supporting the role of probiotics and bioactive peptides and proteins in disease modulation is ever growing. In particular, many studies indicate that fermented milk products have antitumour properties (Kohwi et al., 1978; Aso et al., 1995; Lin and Yen, 1999). Furthermore, a new emerging area where therapeutic potential could be gained by the treatment with probiotics is inflammatory bowel disease (IBD) (Kruis et al., 2004; Ingrassia et al., 2005). However, there is still a lack of knowledge on the mechanisms through which probiotics and biogenics exert their effect. Furthermore, after the identification of a potential probiotic LAB or biogenic, it is essential to thoroughly characterize biological actions. In this manner probiotic strains or biogenics can be precisely chosen to prevent and/or treat specific disease conditions.

Therefore this chapter provides a brief literature review of the role of probiotics and milk proteins as functional food ingredients. The chapter will specifically focus on their current usage in the modulation of cancer and IBD. This will be followed by the research objectives for this project. The following areas had been studied: the role of *Lactobacillus casei* ADA 03 and milk proteins in antimutation, the adhesive properties/mechanisms of the dairy LAB, the cytotoxic, apoptotic and necrotic abilities of dairy derived LAB and milk proteins, as well as the immunostimulatory capacity of LAB and milk proteins, with specific reference to nitric oxide (NO), tumour necrosis factor (TNF-α) and interleukin 6 (IL-6).

1.2 PROBIOTICS – AN OVERVIEW

By convention, the term probiotics refers to LAB belonging to the family Lactobacillacea. Bacteria from this family are gram positive, non-endospore forming, carbohydrate fermenting and as the name implies lactic acid producers. They are acid tolerant, facultatively anaerobic and catalase negative bacteria. Members of the family Lactobacillacea can be subdivided into five genera: *Lactococcus, Streptococcus, Leuconostoc, Pediococcus* and *Lactobacillus* spp. (Kandler and Weiss, 1986).

The use of probiotics in the modulation of disease has had a long history. Roman historian Plinius in 76 A.D. suggested the use of fermented milks to treat gastrointestinal infections. The term probiotic is derived from Greek meaning 'for life'. It was first used by Metchnikoff in its present context. However, it was Fuller who popularized the use of probiotics. He described probiotics as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989). However, this definition has been revised in light of the physiological functions with which have been attributed to probiotics. Fullers' definition stresses the need for viable bacteria and isolates the target of action specifically to the intestinal tract. Probiotic bacteria have been reported to have a variety of effects in other target tissues/organs, such as the breast (Rice et al., 1995), bladder (Aso et al., 1995), reduction of serum cholesterol and lipids (Manaa et al., 2005; Lewis and Burmeister, 2005) and a host of other beneficial health effects. Thus Fuller's definition requires revision.

Naidue et al. (1999) suggest that the definition for probiotics should be 'a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract'. In addition, they suggest that a 'Probiotic Active Substance is a cellular complex of LAB that has the capacity to interact with the host mucosa and may beneficially modulate the immune system independent of LAB's viability'. This definition does not stress the need for viable organisms but acknowledges that there is growing evidence that LAB do not need to be viable nor completely present to have a health enhancing effect.

Probiotics are commonly found in dairy products, specifically fermented milks and yogurt. LAB are able to use milk as a growth medium, producing lactic acid as a major product of fermentation. The lowering of pH (4.3 or 4.5) by LAB aids both in the formation of the curd and in producing an atmosphere not conducive to the growth of vegetative cells and spores surviving heat treatment (Fonden et al., 2003). Fermentation by LAB involves a series of bacteria associated enzymes that can proteolytically cleave casein into free amino acids and peptides, which are not only necessary for bacterial growth, but contribute to the generation of bioactive peptides/proteins (Gobbetti et al., 2002; Law and Haandrikman, 1997).

However, not all bacteria can be classified as a probiotic. There are certain criteria with which a potential probiotic bacterium must fulfill. Probiotic strains should be of human origin, be able to survive that harsh intestinal environment, have adherence and colonization qualities, produce antimicrobial substances, be safe for human consumption and be clinically validated (Lee and Salminen, 1995; Vaughan and Mollet, 1999). In addition to these criteria, others have suggested that probiotics should possess generally regarded as safe (GRAS) status, have desirable metabolic activity, be technologically suitable and act as a potential vehicle for the delivery of recombinant proteins and peptides in a site specific fashion to the human gastrointestinal tract (Collins et al., 1998). Furthermore, each potential strain should be documented and assessed independently and extrapolation due to the inherent differences between strains, extrapolation of data from closely related strains is not acceptable (Salminen et al., 1996; Berg, 1998).

Unfortunately these defining characteristics preclude the inclusion of bacteria from non-human sources as potential probiotics. Indeed, the probiotic bacteria that are currently well characterized and commercially available are *Lactobacillus casei* strain Shirota, *L. rhamnosus* GG and *L. acidophilus* LA1 which are all of human origin (Dunne et al., 2001). However, many studies demonstrate that probiotics of dairy and other food origin have all the qualities listed, except for human origin. Furthermore, studies are now beginning to show that viable probiotic bacteria are not a requirement for physiological action, in fact non-viable cells or portions of cell wall have been found to have strong health enhancing benefits. The use of non-viable cells would be beneficial in diseases where there is a compromised immune system where susceptibility to infection is high. Indeed, several studies in subsequent chapters demonstrate the bioactive qualities of heat killed, cell wall fractions and supernatants isolated from LAB or dairy origin. Thus, the criterion for classification as a probiotic should be re-evaluated and modified to reflect the current scientific findings. The alteration of criterion, would allow for many dairy derived LAB which are well tolerated, able to survive processing and possess health enhancing capabilities to be classified as probiotics.

1.3 MILK PROTEINS – AN OVERVIEW

Milk is an essential food for neonates of all species. However, for humans milk represents a nutritionally sound source of vitamins, minerals and proteins for the entire life span. Milk is composed of 87% water, 3.8% milk fat, 3.3% proteins, 5.0% carbohydrates and 0.7% minerals. Of the 3.3% proteins in milk, approximately 80% of this is casein protein and the remaining 20% is made up of whey proteins (Severin and Wenshui, 2005).

After acid precipitation (pH 4.6) there is a separation of the whey and casein fractions. The isoelectric point of casein is 4.6. At pH 4.6 casein proteins denature and precipitate out of solution forming a visible curd. Caseins are synthesized within the mammary secretory epithelium. Conversely, whey proteins are found in the supernatant fraction after precipitation of casein at pH 4.6. As compared to casein, whey proteins are much more soluble thus stay in solution.

Interestingly, whey has been considered a by-product of cheese/casein manufacture. Due to increased cheese production worldwide, whey and whey protein availability was projected to be well over 90 billion liters/year and 540 million kilograms/year, respectively, at the turn of the century (McIntosh et al., 1998b). Currently, whey is used as starting material for harvesting many important ingredients such as lactoferrin, lactose, proteins, etc. Whey and whey proteins are the source of bioactive proteins and peptides. This represent new and significant opportunities to derive a functional food ingredients from a food processing by- product. In the United States, the functional food market was projected to be greater than $80 billion/year after the turn of the century (Sloan, 1996). This provides a lucrative opportunity for developing functional foods based upon whey protein. Whey protein would be appealing to the consumer because it is not a synthetic product, it is a product naturally present in milk, and in addition to nutrition, it has been scientifically proven confer a specific health advantages.

Encoded into the amino acid sequence of casein and whey there are smaller proteins and peptides. The casein proteins are classified as α-s1 casein, α-s2 casein, β-casein and κ-casein. The two major whey proteins are α-lactalbumin and β-lactoglobulin; lactoferrin, lactoperoxidase and immunoglobulins are also hidden with the native protein structure. Indeed, further within these protein fractions are smaller peptide sequences such as β-casomorphin-7 (Steijns, 2001;Schlimme and Meisel, 1995). For a list of milk derived proteins and peptides and their bioactivities, please refer to tables 1.1 and 1.2.

1.4 HEALTH BENEFITS OF DAIRY DERIVED MILK PROTEINS AND PROBIOTIC LAB

1.4.1. Adhesion of Characteristics of LAB

One of the major criteria for classification as a probiotic is the ability to adhere to and transiently colonize the gastrointestinal tract (Lee and Salminen, 1995; Vaughan and Mollet, 1999; Tuomola et al., 2001). Adhesion aids the bacteria in transient colonization of the gut preventing rapid removal by peristaltic contractions (Johansson et al., 1993;

Alander et al., 1997)). In addition, adhesion would increase the residency time of LAB within the gut, to allow maximum time for probiotic effects. Indeed, in human and animal studies, *L. casei* can be recovered from feces during and up to two weeks after discontinuation of treatment (Goldin et al., 1992; Ling et al., 1992; Saxelin et al., 1991).

It is also well known that adherence confers probiotic bacteria competitive advantage against pathogenic bacteria invading the gastrointestinal microflora. Lee et al., (2000) compared the adhesion of *L. rhamnosus* GG and *L. casei* strain Shirota against pathogen *E. coli* TG1. They found that *L. casei* strain *Shirota* are competing for the same adhesion sites or cell surface receptors of Caco-2 cells; in addition, they demonstrated the *L. casei* strain Shirota adhered more readily and dissociated less easily from Caco-2 cells than pathogenic *E. coli.* Others have demonstrated that *L. rhamnosus* GG competed for adhesion sites on Caco-2 cells against seven strains of *E. coli* and *Salmonella spp.* The competitive inhibition was attributed to steric hindrance (Lee and Puong, 2002).

Forestier et al., (2002) also demonstrated that *Lactobacillus casei* subsp. *rhamnosus* (Lcr35) could inhibit the attachment of pathogenic clinical isolates, enterotoxinogenic *E.coli* (ETEC H10407), an enteropathogenic *E. coli* strain (EPEC2348/69) and *K. pneumoniae* LM21 to Caco-2 cells through exclusion, competition and displacement of pathogens. They suggest that Lcr35 blocks the access of pathogens to tissue receptors by steric hindrance or Lcr35 interacted with Caco-2 cells to modulate the level of mucin produced thus preventing pathogenic adhesion. Furthermore, Ingrassia et al. (2005) demonstrated that *L. casei* DN-114001 was able to inhibit the adhesion of *E. coli* isolated from ileal lesions of patients with Crohn's disease (CD)to Caco-2 cells.

Indeed, *in vivo* studies have shown that *E.coli* (AIEC) strains are highly adhesive and invasive to early and chronic ileal lesions in patients with CD (Darfeuille-Michaud et al., 1998; Boudeau et al., 1999). Non pathogenic *E. coli* strain Nissle 1917 was tested for it's ability to prevent intestinal pathogenic isolate AIEC adhesion and invasion of intestinal epithelial cell line, Intestine-407. *E.coli* strain Nissle 1917 was able to displace attached pathogen and to competitively prevent adhesion of pathogenic AIEC. Furthermore, with the administration of *E.coli* strain Nissle 1917 there was a significant decrease in intestinal cell cytotoxicity, indicating a prevention of invasion as well. These researchers suggest that, *in vivo*, *E.coli* strain Nissle 1917 could form a biofilm around intestinal epithelial cells that would act like an impenetrable layer against pathogenic AIEC (Boudeau, et al., 2003).

The importance of adhesion characteristics does not only apply to prevention of invasion by intestinal pathogens but evidence is beginning to demonstrate that this is also an important parameter for the achievement of health benefits conferred by LAB. Interestingly, Ouwehand et al. (2003) examined the adhesion characteristics of LAB to different diseased tissues as compared to control tissue. They examined six different strains of LAB and their adherence ability to tissue samples obtained from diverticulitis, rectal carcinoma, Crohn's disease and ulcerative colitis patients. They found that there was a strong strain specific and tissue specific adhesion indicating that LAB have differing specificities, and subsequently different degrees of probiotic action for different disease states.

Recent evidence indicates that for LAB to have immunomodulatory activity, adhesion must occur (Granato et al., 2004). Wallace et al. (2003) studied the adherence qualities of ten different strains of LAB. Of the ten strains studied, *L. rhamnosus* R0011 not only demonstrated the highest affinity for binding to HT29 cells but also had the greatest immunomodulating effects, specifically modulating interleukin 8, tumour necrosis factor and transforming growth factor. Conversely, Morita et al. (2002) found that even strongly adhering *Bifidobacterium animalis* was not able to induce the secretion of interleukin 6 and interleukin 8 from Caco-2 cells (Morita et al., 2002).

In *in vitro* experiments, *Bifidobacterium* strain Bb12 and *L. acidophilus* La1 was found to be highly adhesive. Milks fermented with Bb12 and La1 were given to healthy volunteers to drink 120mL, 3 times per day for three weeks. Prior to and after fermented milk treatment, each group was given unfermented milks. They found that volunteers who consumed fermented milk had significantly higher phagocytic activity of blood phagocytes (granulocytes and monocytes). Blood samples taken from volunteers at the end of the study still maintained significant, although lower, phagocytic activity 6 weeks after the end of fermented milk consumption. These researchers identified a strong correlation between bacterial adherence and immunomodulation attributed to Bb12 and La1 (Schiffrin et al., 1997).

Thus these studies illustrate the importance of having direct bacterial cell adhesion with target cells as a major criterion for LAB being labeled as a probiotic. Unfortunately, the measurement of adhesion *in vivo* is confounded by many factors and is quite costly, thus in *in vitro* adherence assays that have strong correlation with *in vivo* findings, have been developed to screen potential probiotics for adhesion qualities (Blum et al., 1999; Dunne et al., 2001). In addition, these *in vitro* screening methods allow a direct method of determining factors or mechanisms involved in bacterial attachment to intestinal epithelial cells.

Chapter 2 describes the adhesion of eight dairy isolated LAB to HT29 colon cancer cell line as measured through light and scanning electron microscopy. In addition, we applied several chemical and physical treatments to determine what types of adhesins are involved in bacterial attachment. In particular, we have examined the adhesion characteristics and mechanisms of *L. casei* ADA03. Previously, experiments performed in our laboratory demonstrated efficacy of *L. casei* ADA 03 in conferring antitumour effects on Morris hepatoma cancer cell line (Macleod, 1990) and bactericidal effects against *Helicobacter pylori* (Wendakoon and Ozimek, 2002). Thus it is of interest to us to determine if this bacterial strain is highly adhesive and what factors are involved in its binding.

1.4.2. Antitumour Activity of Probiotic LAB

The term cancer, first used by Hippocrates and other Greek physicians (500 BC to AD 200), is derived from the Latin word for 'crab'. They attributed cancer to be similar to a crab, due to the crab-like appearance of a variety of carcinomas that spread and invaded surrounding tissue ultimately resulting in death of the patient. Later, terms like neoplasm were coined by Galen (AD 200) and the term metastasis, Greek word meaning to change places, by other Greek physicians (World Cancer Research Fund, 1997).

Clinically, cancers represent over one hundred diseases; however, all these diseases are created from similar defects in cellular function and alterations to cell genes. Thus, ultimately cancer can be described as a disease of altered gene expression (Ruddon, 1995). Cancer is a complex process, involving derailment of many of the body's protective mechanisms. There are three defined stages of cancer development: initiation, promotion and progression. The development of cancer treatment regimes often attempts to intervene and inhibit cancer development through impeding these stages of cancer.

Initiation includes a series of events whereby cells come in contact with carcinogens that induce an alteration or mutation in the DNA of the cell. Although initiation of DNA damage does not always result in cancer, this mutation can be inherited by daughter cells and increases the potential for neoplastic growth. Mutations occur at relatively high rates within the body, however, DNA repair mechanisms are constantly surveying the body for such mutations and initiate corrective actions.

However, if DNA repair systems are unable to repair damage, the mutated cell will persist and replication will occur resulting in a population of damaged daughter cells. This stage is referred to as promotion stage and the cells are now referred to as a neoplastic cell. Initiation of mutation and promotion of that mutation may take many years to discover. Neoplastic cells may stay dormant for many years, until some event such as a mutation to tumour suppressor gene or proto-oncogene initiates progression, unchecked growth and ultimately metastasis (Ruddon, 1995; World Cancer Research Fund, 1997).

It has long been suspected that dietary factors contribute to the development of cancer. Epidemiological studies of migrant populations suggest a strong link between diet/environmental factors and cancer development. In a report by Parkin et al. (2005), Asian migrants showed increased colo-rectal cancers after migrating to countries with western diets. McMichael and Giles (1988) studied the rates of breast cancer incidence in female Italian migrants in Australia. They found that breast cancer incidence increased with longer duration of residency, suggesting a change in environmental factors contributed to a change in breast cancer frequency.

Even though epidemiological studies indicate a strong correlation between diet and cancer causation, there are others that indicate consumption of particular foods can decrease the incidence of cancer. Many studies have reported the benefits of diets high in grains, fiber and green leafy vegetables in the prevention of cancer (Wark et al., 2005;

Robertson et al., 2005; Hermann et al., 2002; Steinmetz and Potter, 1996). Moreover, epidemiological studies have demonstrated an inverse association between cancer development and yogurt consumption (Senesse et al., 2002; as reviewed by Norat and Riboli, 2003). Case Control studies have identified a positive effect of dairy product consumption against colon cancer. Young and Wolf (1988) found that fermented milks consumption significantly decreased the incidence of cancer in the proximal but not distal colon. However, Peters et al. (1992) found that yogurt, but not fermented milks protected against colon cancer. Furthermore, two studies from France supported the findings of Peters and colleagues. They found that the consumption of yogurt decreased the incidence of large colon adenomas as compared to small adenomas (Boutron-Rualt et al., 1999a; Boutron-Rualt et al., 1999b).

However, other studies have not been able to identify a significant effect of yogurt/fermented milk intake and cancer reduction. In two large prospective studies, the Health Professionals Follow-up Study and the Nurses Health study, fermented food intake (yogurt, cottage cheese, hard cheese and sour cream) did not significantly decrease the incidence of colorectal cancer (Kampman et al., 1994a). Nor were they able to identify a link between daily intake of fermented milk products and yogurt intake in the Netherlands (Kampman et al., 1994b).

Thus epidemiological studies demonstrate conflicting results. Some studies establish a link between dietary intake of fermented dairy products and colon cancer, while others do not. This could be due to the classification of fermented dairy products in questionnaires; not all cultures used to ferment dairy products have probiotic potential. Moreover, epidemiological studies can only illustrate correlations. They cannot determine causation.

Animal studies have demonstrated a relationship between colon cancer development and ingestion of fermented milk, yogurt and preparations with LAB. Abd El-Gawad (2004) demonstrated consumption of dairy and soy based yogurt supplemented with *Bifidobacterium lactis* Bb-12 and *Bifidobacterium longum* Bb-46 were able to inhibit the growth of Ehrlich ascites tumour cell in mice.

Tavan et al. (2002) studied the effect of *Bifidobacterium animalis* and *Streptococcus thermophilus* consumption on the formation of heterocyclic aromatic amine (HAA) induced colon carcinogenesis in mice. HAA is the compound generated during the cooking of proteins at high temperatures. They specifically used a mixture of three HAAs commonly present in the western diet, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3-methylimidazo[4.5-f]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ). Pretreatment of rats, with milks containing *Bifidobacterium animalis* and *Streptococcus thermophilus* significantly decreased intermediate biomarkers of carcinogenesis; there was a significant reduction in DNA lesions and mutations in the colon and a significant increase in undamaged cells as well. They concluded that milks fermented with these two bacterium might be able to interrupt the cancer process at the initiation stage of colon carcinogenesis. *In vitro* studies confirm the binding of heterocyclic aromatic amines by LAB (Orrhage et al., 1994; Sreekumar and Hosono, 1998). Reddy and Rivenson (1993) found that oral administration of 0.5% *Bifidobacterium longum* decreased the incidence of liver tumours and colon tumours induced by 3-amino-1-methyl-5H-pyrido[4,3,6]indole (Trp-P2)., 80% and 100 % respectively, in rats.

Furthermore, Zsivkovits et al. (2003) studied the protective effect of four strains of LAB (*Lactobacillus bulgaricus* 291, *Streptococcus thermophilus* F4, *Streptococcus thermophilus* F3, and *Bifidobacterium longum* BB536) currently used in yogurt production on the induction of colon and liver tumours in rats by mixtures of HAA commonly found in fried beef and chicken. They used a technique that measures DNA migration (COMET ASSAY). Previously researchers have showed that a reduction in HAA-induced DNA migration by dietary compounds is paralleled by a reduction in the formation of preneoplastic lesions in the colon and liver (Kassie et al., 2002; Kassie et al., 2003). Zsivkovits and colleagues (2003) found that all bacterial strains were able to completely prevent fried beef derived heterocyclic amine induced DNA damage in the colon and the liver, however no effect was observed for chicken amines. In addition, they found that administration of *L. bulgaricus* 291 up to 12 hours prior to beef HAA administration conferred a significant protective effect against DNA damage.

In 1,2-dimethylhydrazine (DMH) induced colon cancer of rats, de Moreno de Leblanc and Perdigon (2004), found administration of yogurt (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) did not have any inhibitory effect on the initiation stage of cancer development. It only delayed tumour onset. However, in rats fed yogurt after DMH tumour induction, there was complete amelioration of disease; indicating that yogurt can inhibit tumour promotion and progression in DMH induced cancer in rats. Balansky et al. (1999), found that supplementation of rat diets with freeze dried milk (FFM.B144) fermented with *Lactobacillus bulgaricus* strain LBB.B144 inhibited DMH induced colon carcinogenesis in rats. Interestingly, not only was there a decrease in tumour incidence but an increase in the frequency of benign and highly differentiated malignant tumours was also observed.

Onoue et al. (1997) studied the effect of *Bifidobacterium breve* YIT 4010 on DMH induced tumour incidence. Their study was divided into two parts. In the first part of the study, they orally inoculated germ free rats with 10 strains of intestinal bacteria (GB) (*Bacteroides* spp., *Clostridium* spp., *Enterococcus faecium* and *Escherichia coli*) or with fecal bacterial suspension from normal conventional rats (Cvd) or left germfree (control). They found in GB rats there was a significantly higher number of aberrant crypt foci (ACF), ACF with four or more crypts, and higher mean number of aberrant crypts per focus than both the control and the Cvd group. In the second part of the study, in GB mice they also added *Bifidobacterium breve* to the inoculation mixture. They observed a significant suppression in the previous three mentioned parameters. This indicates that *Bifidobacterium breve* is able to suppress DMH induced colon carcinogenesis. Furthermore, Perdigon et al. (1998) found that yogurt administration not only prevented DMH induced tumours but also reduced the inflammatory immune response and the amount of β-glucuronidase in the intestinal fluid.

β-Glucuronidase, azoreductase and nitroreductase are fecal bacterial enzymes that can convert indirect carcinogens into direct acting carcinogens; increased expression is considered a marker for carcinogenic activity in humans (Spanhaak et al., 1998). Through alteration of the gastrointestinal microflora, these bacterial enzymes can be up regulated or down regulated. Animal studies show that administration of LAB can substantially reduce these fecal bacterial enzymes activities (Goldin and Gorbach, 1983; Choi et al., 2005). Indeed, Djouzi et al. (1997) demonstrated that milk fermented with *Lactobacillus casei* and yogurts prepared with both *L. casei* and standard yogurt cultures were not only able to decrease fecal bacterial enzyme activity, but also increased the production of butyric acid. Butyric acid is a short chain fatty acid that selectively induces cancer cells into apoptosis via mitochondrial destabilization.

Furthermore, Kulkarni and Reddy (1994) and Abdelali et al. (1995) found that pretreatment of rats with *Bifidobacterium* not only decreased β-glucuronidase activity but also significantly inhibited development of azoxymethane (AOM) and DMH induced cancer, as measured by decreased number of aberrant crypt foci and crypt multiplicity. Aberrant crypts are structures resulting from overgrowth of the colonic crypts that can be observed to be enlarged and elevated relative to normal crypts (Brady et al., 2000). Aberrant crypts are accepted as preneoplastic endpoints of colorectal cancer (Saikali et al., 2004).

However, in a study by Rice et al. (1995) no anticancer benefits could be attributed to diets supplemented with LAB. In their study mice were divided into two groups. One group was used to study the initiation stage and the other group was used to study the promotion stage of cancer progression. Experimental diets containing varying amounts of yogurt powder (culture free), *Bifidobacterium* or *L. acidophilus* were used. For the initiation study, mice were given one of the experimental diets 4 weeks prior and during the six weeks of 7,12-dimethylbenz[a]anthracene (DMBA) tumour initiation. For the promotion studies, rats were fed a standard diet prior to and during DMBA tumour initiation. One week after the end of DMBA tumour initiation, one of the experimental diets was administered until the termination of the diet. They did not find any statistically different rate of tumour incidence between any of the treatments. Furthermore, McCarthy et al. (1997) found that the same treatments were not able to inhibit DMBA induced skin tumour development. However, interestingly for the study performed by Rice et al. (1995), although not significant the diet with the highest concentration of yogurt powder had the lowest tumour incidence in the initiation study.

This could indicate that bioactive antitumour products were produced during fermentation. However, these researchers did not study this parameter.

Thus these *in vivo* experiments demonstrate that LAB does have antitumour capabilities against experimentally induced cancers. Some studies demonstrate that LAB can mount an effective antitumour response at several stages of tumour development. However, this response varies depending upon the time of administration, type of carcinogen being used and more importantly the strain of LAB being tested and the origin (human or dairy). Thus this strengthens the need to have well characterized strains of bacteria.

Unfortunately, cancer is a very multifaceted disease, where development does not necessarily follow a linear course as observed in controlled animal studies. Animal studies provide more direct evidence of action, whereas human clinical trials are more indirect and circumstantial, however, human studies are important to refute or confirm findings from animal studies. Humans are considered to be the ideal subjects for testing and monitoring outcomes of treatments. For obvious ethical reasons it is more difficult to obtain approval to perform human clinical trials. For this reason human studies must choose surrogate end points for cancer risk and development instead of tumour development. However, identification of ideal surrogate end points such as polyp recurrence and occurrence can involve very invasion procedures. Thus the majority of studies are performed on health subjects where indirect measures of colon cancer development are measured. Parameters such as fecal water toxicity (Van Loo et al., 2005), urinary mutagenicity (Hayatsu and Hayatsu, 1993), intestinal bacterial enzymes and fecal bile acid concentrations and contents are measured (Saikali et a., 2004; Brady et al., 2000; Hayatsu and Hayatsu, 1993). However, there are only a limited number of human studies and probiotic actions available.

In a very early clinical trial, healthy humans were administered milks (2 glasses × 250 mL/day for 4 weeks) fermented with either human isolate *L acidophilus*-N2 (2×10^6/mL) or *L. acidophilus* NCFM (2×10^6/mL). The fermented milks significantly lowered fecal bacterial enzymatic activity of β-glucuronidase, nitroreductase and azoreductase. This decrease was generally observed 20-30 days into fermented milk administration and was maintained for between 20 and 30 days after the end of administration (Goldin and Gorbach, 1983).

Spanhaak et al. (1998) studied the effect of milk fermented with *Lactobacillus casei* strain Shirota in a western type of diet in normal healthy individuals. Twenty subjects were divided into a treatment group or the control group. Treatment subjects were given 3 × 100 mL /day of *L. casei* strain Shirota fermented milk, while the control group received the same volume of unfermented milk. The study period lasted for 4 weeks and was preceded and followed with 2 week washout periods. They observed a significant decrease in β-glucuronidase and β-glucosidase activities; whereas urease and tryptophanase activities were not altered. Interestingly, this study also demonstrated that there was no statistically significant alteration in various immune parameters after the ingestion of *L.casei* strain Shirota fermented milk.

In a recent study, 64 healthy females were given a yogurt (2 × 150mL/day) containing *L. rhamnosus* GG, *L. rhamnosus* GG and rye fiber or a placebo yogurt and rye fiber. Fecal β-glucuronidase, nitroreductase and glycocholic acid hydralase activities were all significantly lower in the *L rhamnosus* GG supplemented yogurts regardless of fiber content. Fiber did not seem to affect fecal enzyme activity (Ling et al., 1994).

Furthermore, Hayatsu and Hayatsu (1993), studied the effect of *L. casei* (freeze-dried cells; 10^{10} cells/g × 1-5 g; 3×/week) consumption on urinary mutagenicity induced by ingestion of fried ground beef (250 g/day), through the measurement of urinary mutagenicity. They found that ingestion of *L. casei* decreased fried ground beef induced mutagenicity by 47.5%.

In a recent study 13 patients who refused chemotherapy or radiation therapy, with various stages and forms of metastatic cancer of diverse origin, were given *Bacillus oligonitrophilus* KU-1 as a form of cancer treatment. The difference between predicted and actual life span were measured; in addition, a change in oncomarker, cancer antigen (CA) was measured. *Bacillus oligonitrophilus* KU-1 was orally administered in varying doses (10%, 1%, 0.1% (v/v) in 50-400 mL/day) was diluted into warm milk, juice or soda. They found that all patients had a significant reduction in CA and more importantly, the administration of *Bacillus oligonitrophilus* significantly impacted life prolongation. All patients on this treatment exceed expected survival times (Malkov et al., 2005). Although *Bacillus oligonitrophilus* shows immense potential as a biotherapeutic agent in multiple forms of cancer treatment, it is important to note that these were uncontrolled observations.

Based upon current literature available on probiotic and prebiotic effects in cancer, the European Union sponsored SYNCAN project, a large-scale comprehensive study involving *in vitro, in vivo* and human interventions is underway (van Loo et al., 2005). The *in vitro* and *in vivo* studies have shown that the symbiotic (Synergy®/*Lactobacillus rhamnosus* GG/*Bifidobacterium lactis* Bb12) has cytotoxic and antigenotoxic activity in HT29 and Caco-2 cells and reduces AOM-induced carcinogenesis in rats. However, the human clinical trial results have not yet been published.

These human trials and animal trials demonstrate an association between probiotic ingestion and the prevention of cancers. However, it is important to note that many of these human clinical trials track consumption and changes in cancer parameters for relatively short periods of time. Epidemiological studies demonstrating a strong link between cancer development and fermented dairy product consumption track consumption over longer periods of time.

Regardless, the use of probiotics as chemotherapeutic adjuvants is promising. However, the LAB that are currently classified as probiotics are from human origin. There is a need to isolate and characterize LAB with potential probiotic activities from other sources. In particular, LAB isolated from dairy products seem to be ideal candidates because they already have GRAS status, are currently being used in dairy product production and have already been found to have a variety of biological effects. Moreover, the concept of a therapeutic agent present in a food product shifts the health paradigms currently in place from treatment of disease to prevention of disease. So we decided to characterize and determine *in vitro*, the possible effects that ingestion of dairy derived LAB can have.

1.4.3 Antitumour Activity of Dairy Derived Milk Proteins

Bioactive compounds are defined as a substance that has an effect on living tissue. Bioactive compounds can be isolated from many different food sources; however, the majority of the bioactive peptides are currently derived from milk (Table 1.1 and 1.2). There are many bioactive peptides or biogenics encrypted within the native/parent protein structure of milk, however these remain biologically inactive until they are released from the native protein structures. These proteins can be liberated from milk through hydrolysis by gastrointestinal tract acidity or LAB associated enzymatic activity, acid precipitation of casein or mechanical processing (Law and Haandrikman, 1997; LeBlanc et al., 2002). Once released, these proteins can have vast biological action.

As discussed above, some epidemiological studies demonstrate an inverse correlation between fermented milk consumption and cancer development. It is generally assumed, that the LAB used to ferment the milk is the anticancer agent. However, some researchers have demonstrated that LAB possess a complex proteolytic series of enzymes that can digest milk casein into smaller bioactive peptides (LeBlanc et al., 2002). This proteolytic system is comprised of a proteinase involved in the initial cleavage of milk casein, then peptidases that can hydrolyse the larger peptides into smaller more biologically active ones (Law and Haandrikman, 1997). In addition, processing liberates many bioactive peptides from their native protein structure.

Furthermore, epidemiological studies also suggest that there is an inverse relationship between unfermented milk ingestion and cancer incidence. In a 25 year study, following originally cancer free women, a significant correlation was found between milk intake and the incidence of breast cancer (Knekt et al., 1996). Mettlin et al., (1990) found a significant reduction in the risk of developing cancers in the oral cavity, stomach, colon, rectum, lung, bladder, breast and cervix with the higher consumption of milk.

Although casein is the predominant protein in milk, whey protein fractions have been found to have more biological activity. Whey protein has been more extensively studied for their inhibitory effects in cancer induction. Indeed, the use of whey protein in disease prevention is not a new concept. Whey has been used in the prevention and treatment of disease as a part of folk medicine. According to McIntosh et al. (1998b) documentation by Dr. Julius Ceasar Baricellius of the health benefits of whey date back to the 17th century in Italian literature. Whey was used to treat acute septic conditions and gastrointestinal infections.

Diets supplemented with whey, casein, soybean or red meat were compared for their effect on DMH induced tumour development (McIntosh et al., 1995). Diets were supplemented with 20g/100g with each respective protein. Diets containing whey and casein conferred significant protection against DMH induced tumours. Furthermore, intracellular concentrations of glutathione were significantly higher in the animals fed whey and casein. This could be due to the high concentration of cysteine present in the milk proteins, particularly in whey proteins; cysteine is a precursor for glutathione synthesis (McIntosh et al., 1995). However, McIntosh et al. did not observe significant antitumour activity associated with casein diets. Furthermore, Papenburg et al. (1990) observed a significantly lower incidence of DMH induced tumours in mice fed whey protein in comparison to casein and standard mouse diets.

Ironically in an experiment designed to measure the anticancer potential of legumes, the anticancer potential of casein diets was observed (McIntosh et al., 1998a). McIntosh (1998a) compared diets consisting of casein and chickpeas, a source of soluble dietary fiber, for the tumour inhibitory capacities on DMH induced colon carcinogenesis in rats. The diets were supplemented with wheat and compared against a control diet consisting of casein and starch. They found that diets containing casein and wheat conferred a significantly higher degree of protection against tumours induced by DMH. There was a 20% reduction in tumour incidence as compared to the control diet and a 40% reduction as compared to the diet containing chickpeas and wheat (McIntosh et al., 1998a).

Furthermore, in azoxymethane induced colon cancer in rats, the administration of diets containing 32%, 16% and 8% whey protein or red meat protein (barbequed kangaroo meat) was measured for their ability to inhibit colon cancer development, as measured by aberrant crypt foci. The diet containing 32% whey protein had significantly less development of aberrant crypt foci in the proximal colon as compared to the 16 and

32% meat groups (Belobrajdic et al., 2003). Indeed, Hakkak et al. (2001) found that in rats fed a whey containing diet, there was a 40% lower tumour incidence and 32-42% less incidence of invasive adenocarcinoma in the colon as compared to rats fed a casein diet in azoxymethane induced colon cancer (Hakkak et al., 2001).

In another study, Hakkak et al. (2000) observed a significantly lower incidence of mammary tumour incidence with diets containing whey protein. They also demonstrated that whey protein was much more effective than soy protein supplemented diets at reducing the incidence of mammary tumours.

These results suggest a strong correlation between dietary whey protein supplementation and the inhibition of cancer development. However, these studies were all performed on young rats. Animals of all species are at higher risk of developing cancer as they age; studies determining the role of anticancer food components in mature subjects is lacking.

McIntosh et al. (1998b) examined the influence of dietary meat (barbequed red meat) and whey protein on chemically induced tumours. They studied this effect in mature rats (6-11 months old) because this better reflects the stage in life at which greatest susceptibility to colon cancer occurs. In this study, rats were administered standard diets supplemented with whey protein and barbequed beef (16-20% total protein). Although statistically insignificant, they found that diets supplemented with whey protein had a lower incidence (25% less) of DMH induced tumours than barbequed beef diets. However, they concluded that the age of the rat did not significantly affect disease outcome.

In addition to studying the effects of whole casein and whey protein, studies have been performed to determine the anticancer properties of individual proteins found in milk. McIntosh et al (1998b) supplemented individual protein fractions into young rats diets. Interestingly, they found that diets supplemented with lactoferrin and β-lactoglobulin as compared to standard soy diets, conferred significant anticancer protection against DMH induced colon carcinogenesis. Rates of tumour incidence were similar to those of whey protein supplemented diets. Indeed, Sekine et al. (1997) found that rat diets supplemented with 0.2 or 2% bovine lactoferrin had significant decreases in azoxymethane induced colon adenocarcinomas. In the 0.2% group, the incidence of colon adenocarcinomas was reduced to 25% and in the 2% group to 15%, as compared to a control group where the incidence of adenocarcinoma was 57%. Interestingly, Tsuda et al. (1998) found similar results, however, they also found that a bovine lactoferrin hydrolysate had similar activity to the two different concentrations of lactoferrin reported by Sekine et al. (1997), whereas lactoferricin had only 10% antitumour activity against azoxymethane induced colon carcinogenesis. These last results are surprising, because lactoferricin has higher binding capacity to iron than lactoferrin. Thus, lactoferricin should confer a higher degree of antitumour activity.

β-Lactoglobulin and lactoferrin are rich in sulphur amino acids (Walzem et al., 2002). It is suggested that sulphur rich amino acids, such as methionine and cysteine, can stabilize DNA through their influence on DNA methylation status and thus influence cancer development (Rogers, 1993). Indeed, McIntosh et al. (1998b) suggests that sulphur amino acid rich proteins, such as β-lactoglobulin and lactoferrin, reduce colon tumours and tumour precursors through the stabilization of DNA by providing biologically available cysteine and methionine. Weinberg (1996) suggests that lactoferrin may confer antitumour properties through its iron-binding properties since free iron may act as a mutagenic promotor by inducing oxidative damage to nucleic acid structure.

Others have suggested that whey proteins are potential anticancer agents due to its contribution of cysteine rich residues to glutathione synthesis. Within glutathione, cysteine acts as an active agent by preventing oxidation and tissue damage. The accumulation of intracellular reactive oxygen species is associated with the development of cancer (Nelson et al., 2001). Furthermore, the depletion of intracellular glutathione is followed by an increased accumulation of intracellular reactive oxygen species (Esteve et al., 1999). Prostate tissue is highly susceptible to oxidative stress and consequently to cancer development. Interestingly, Kent et al. (2003) found increased intracellular glutathione concentrations in human prostate epithelial cell line (RWPE-1) after treatment with hydrolysed whey protein isolate. In addition, the same authors reported that whey protein isolate was able to inhibit oxidant-induced cytotoxicity. In contrast, Kent et al. (2003) found that hydrolysed sodium caseinate, which is low in cysteine, did not significantly elevate intracellular glutathione. This suggests a role for cysteine rich amino acids in cancer prevention. Indeed, the consumption of cysteine rich whey proteins in HIV patients has been shown to increase the plasma glutathione concentrations in humans (Micke et al., 2001).

However, once cancer develops glutathione concentrations are much higher in cancer cells than normal cells; the elevated glutathione levels is thought to confer chemotherapeutic resistance to cancer cells (Kennedy et al., 1995). In human hepatoma cancer cell line Hep G2, the administration of Immunocal, a patent-protected whey protein isolate (contains serum albumin, lactoferrin and α-lactalbumin), augmented the cytotoxic activity of anticancer drug baicalein by inducing increased levels of apoptosis; this increase in apoptotic cells was associated with the depletion of glutathione in Hep G2 cells (Tsai et al., 2000). Furthermore in other studies the sparing effect of Immunocal on normal cells versus cancerous cells has been demonstrated (Bounous and Gold, 1991; Baruchel and Viau, 1996).

Immunocal was used in the treatment of patients with metastatic carcinoma. There were seven patients enrolled in this study (five had metastatic carcinoma of the breast, one of the pancreas and one of the liver). These patients were put into a treatment regimen where they received 30 g of Immunocal daily for six months. Interestingly at the end of the study, four of the patients showed stabilization or regression of tumours as well as a significant reduction in lymphocyte levels. Unfortunately, three of the patients did not show any improvement in disease status after treatment with Immunocal. Regardless, the researchers suggested that whey protein concentrate was able to decrease tumour cell glutathione concentrations, thus rendering them more susceptible to chemotherapeutic intervention (Kennedy et al., 1995).

The previously discussed studies illustrate the potential role that milk proteins/peptides can play in cancer prevention, in particular with whey protein. Since it is considered to be a by product of cheese production, the development of therapeutic products is a desirable option from an industrial, commercial and health care perspective. This provides a tremendous and inexpensive resource with which bioactive compounds are obtained. The other major protein present in milk, casein is retained in the dairy products thus are consumed as part of a food product. Several studies have demonstrated the anticancer benefits of casein ingestion, however, it is not as well characterized for it's possible antitumour effects as whey. The concept of a biotherapeutic agent that is already available in a food item is an extremely attractive prospect to consumers who are becoming more health conscience, to the commercial sector that is constantly deriving new angles to market functional foosd and to health care sector, where the cost of treating cancer is tremendous. Moreover, industrial scale processes are currently in established for the recovery of biologically active whey and casein proteins. Thus there is a need to characterize the bioactivity of dairy derived casein proteins.

Many studies have demonstrated the positive effect of milk derived proteins and in modulating tumour outcomes, however, their mechanisms are not well known. Animal studies provide us a direct method to measure the outcome of treating with potential anticancer agents. However, due to the inherent complexities of complete animal systems there are confounding factors that preclude the ability to determine mechanisms of actions. *In vitro* conditions allow for control of these confounding variables thus allowing elucidation of mechanisms of actions.

1.4.4 Inflammatory Bowel Disease

A wide variety of pathological conditions, such as campylobacter enteritis, salmonellosis, shigellosis, amebiasis, or viral enteritis result in inflammation of the colon. However, while these conditions may be associated with inflammation of the colon, the term inflammatory bowel disease (IBD) by convention refers to two conditions, ulcerative colitis (UC) and Crohn's disease (CD). IBD is generally associated with diminished bowel function, involving both digestive and absorptive processes. Diarrhea and/or steatorrhea (excessive fat in the feces) are common. Blood may also be found in the feces with severe inflammation or ulceration of deeper areas of the gastrointestinal mucosa.

The mechanisms responsible for initiation, perpetuation and pathogenesis of IBD remain elusive, however, it is predominantly theorized that IBD may result from abnormal host responses to some members of the intestinal flora, from a defective mucosal barrier or an alteration in the balance between putative species of protective vs. harmful bacteria, a concept termed dysbiosis (Tamboli et al., 2004). Indeed, patients with UC, CD and pouchitis have abnormal intestinal flora type of bacteria and their concentrations (Bennet and Brinkman, 1989; Borody et al., 1989). Furthermore, there are increasing concentrations of gram-negative anaerobes, particularly *Bacterioides* spp in CD (Van de Merwe, 1988) and UC (Matsuda et al., 2000; Ohkusa, et al., 2002) and *Clostridium perfringens* in pouchitis (Ruseler-van Embden et al., 1994; Duffy et al., 2002). Concurrent with this increase in harmful bacteria, others have found reductions in the beneficial bacteria in IBD (Favier et al., 1997; Ruseler-van Embden, 1994).

Treatment of IBD during the first third of the 20th century included 'slop diets', opium, tincture of hammamelis, rectal instillations of boric acid or kerosene, thiouracil drugs, copper sulphate, liver extract, artificial fever, azocloramid and interestingly milk soured with LAB (Kirsner, 1998). Although the concept of treating IBD with milk soured with LAB seems archaic, there has been a resurgence of interest in using a variety of strains of LAB for improving intestinal health. Indeed, a study in Australia on children with IBD determined that 72% of those surveyed (n=46) were using complementary and alternative medicines in conjunction with standardized treatment. Furthermore, of this group 78% reported using probiotics in the treatment of IBD. However, there were mixed results; some parents reported that the use of probiotics was effective or very effective, others reported the agent as partially effective and some even said the alternative treatment was completely ineffective at controlling IBD in their children (Day et al., 2004). Regardless, there is still evidence indicating the potential uses of probiotics in the treatment of IBD.

The distal ileum and colon have the highest concentration of luminal bacteria and are often the sites of inflammation in IBD. The inflamed mucosa is constantly exposed to bacteria, and the inflammation often occurs at places with high bacterial concentrations. This demonstrates a possible etiological link between intestinal microorganisms and inflammatory bowel disease (Brown, 1995; Macpherson et al., 1996; Sellon et al., 1998; Von Wulffen et al., 1989). However, it is not known whether the bacteria provide the initial trigger of the disease or simply act as amplifiers of inflammation initiated by some other insult. An adverse intestinal microflora may cause increased intestinal production of toxic bacterial products or changes in short-chain fatty acid levels; which in effect may cause inflammation (Malchow, 1997). Involvement of pathogenic or potentially pathogenic bacteria in IBD has long been suggested because, among other reasons, the inflammatory response resembles that in infectious bowel disease (Fedorak and Madsen, 2004).

Hyper reactivity of the intestinal mucosa to ubiquitous antigens produced by the intestinal microflora has been implicated in the perpetuation of IBD. Indeed, evidence shows that there is a breakdown in intestinal tolerance to the normal bowel flora in active IBD (Brown, 1995; Sellon et al., 1998; Von Wulffen, 1989). Strains of *E.coli* isolated from patients with UC produce more enterotoxins and hemolysins and degrade mucin more efficiently than strains from healthy controls (Von Wulffen, 1989).

All of these factors point to the idea that dysfunction of the intestinal mucosal barrier and dysbiosis contribute to inflammatory disease. However, despite the numerous therapeutic improvements that have occurred since the early 1900's, particularly in the field of antibiotics and anti-inflammatory drugs, IBD and its consequences remain a major clinical problem. To complicate the clinical picture, there has been a dramatic increase in the incidence of antibiotic-resistant microbial pathogens. A major concern within the scientific community is that industry will no longer be able to develop effective antibiotics at a rate sufficient to compete with the development of microbial resistance to old antibiotics. There is a renewed interest in the possibility of deliberately introducing beneficial microorganisms to humans as an alternative or adjunct to antibiotic therapy in gastrointestinal disorders.

Therefore, several therapeutic strategies have been devised; these will be discussed in the following sections. Briefly, new therapeutic modalities include reduction or dilution of bacterial components in the intestine by antibiotics or intestinal lavage, inactivation of inflammatory bacterial products, and reconstitution or modulation of intestinal microflora through the use of probiotics have been researched and show some promise in the treatment of IBD.

1.4.4.1 Animal Studies

There is increasing experimental evidence to support the involvement of intestinal bacteria in the pathogenesis of CD. In genetically engineered interleukin-10 (IL-10) knockout mice, spontaneous colitis will only develop in the presence of luminal bacteria; colitis does not generally develop when raised under germfree conditions. IL-10 knockout mice housed in conventional conditions develop a patchy, chronic colitis that is similar to human CD, suggesting that luminal bacteria play a role in the initiation of gastrointestinal inflammation (Sellon et al., 1998; Kennedy et al., 2000a; Kennedy et al., 2000b).

Indeed, Madsen et al. (1999) examined the effect of repopulating the gut of IL-10 knockout mice with *Lactobacillus reuteri.* In 2 week old mice, histological injury is not yet present, however, there is an increase in colonic mucosal adherent and translocated aerobic bacteria along with decreased *Lactobacillus* sp. bacteria present within the colon as compared to control mice. Thus, they attempted determine if increasing *Lactobacillus* spp. would effectively normalize the colonic microflora of IL-10 knockout mice. Mice were given a single enema of *L. reuteri* at one week of age; daily rectal swabbing with *L.reuteri* followed this. When 4 week old mice were examined, *Lactobacillus* spp. were restored back to the level of the controls, furthermore, *L. reuteri* administration attenuated the development of colonic histological injury characteristic in IL-10 knock out mice as well as a normalization of adherent and aerobic translocated bacteria.

Schultz et al. (2002) studied the effect of oral probiotic administration of *L. plantarum* 299V (L299V) on colitis in 10-12 week old IL-10 deficient mice. The mice were randomized into three groups: a) simultaneous administration of L299V and specific free pathogen (SPF: free of *Helicobacter* spp.), b) precolonization, where mice were given L299V two weeks prior to SPF exposure, and c) administration of L299V to mice with pre-existing colitis. They found that there was a significant inhibition of colitis development in the groups with established colitis and the precolonization group, suggesting that L299v is effective at treating and preventing colitis. Interestingly, no effect was observed in the simultaneous exposure group. Schult et al. (2002) suggest that L299V was ineffective at preventing colitis development during spontaneous exposure because the intestinal environment was exposed to a large bacterial load, the pathogenic and inflammatory bacteria were more adept at colonization than the probiotic.

Conversely, not as positive results were obtained using an alternate animal model of colitis. The effect of the probiotic *Lactobacillus plantarum* species 299 (LP299) on the severity of colitis and intestinal permeability was investigated using the hapten model (Kennedy et al., 2000a). The hapten induced model of colitis in mice, has been well characterized and has clinical, biochemical and pathological similarities to CD. Furthermore, the model is associated with a significant increase in the numbers of aerobic gram-negative bacilli in the colon, a pattern that is similar that seen in to microflora disturbances in human IBD. There were two treatment protocols with LP299 administered in an oat fibre suspension (LP299/OF) containing at least 10^9 colony forming units of LP299 or oat fibre (OF) suspension alone. LP299, a bacterium often cultured from the human intestine, was selected for this study since it is known to survive passage through the gut and adhere to the mucosa. There were five groups of animals; colitis receiving no treatment (n=16), colitis treated with LP299/OF (n=16), colitis treated with OF only (n=16), and non-colitic controls receiving either LP299/OF (n=8) or OF (n=8). They did not find that the addition of either LP299 or OF improved the intestinal permeability or the severity of the colitis symptoms. This may be due to the fact that this hapten model of colitis causes inflammation that is too severe in nature for any positive effect of the probiotic to be observed.

The acetic acid-induced model of colitis is characterized by increased neutrophils infiltration and a decrease in mucosal permeability, all characteristics of colitis. Fabia et al. (1993) demonstrated that intracolonic administration of *L. reuteri* L2LC (5 mL; 7×10^7 CFU/mL) immediately after acetic acid induction of colitis prevented the development of colitis. However, *Lactobacillus* HLC was ineffective a preventing colitis.

Pouchitis is a common side effect of IBD after surgical resection. Okada et al., (1999) studied the influence of intestinal flora on the healing process in intestinal anastomosis. Five groups of rats were studied: germ free, conventional, mono-contaminated with *Lactobacillus acidophilus* La5 or *Escherichia coli* X7 and ex-germ free (conventionalized). These animals underwent ileal and colonic resections followed by anastomosis. They found that the animals mono-contaminated with either *L.acidophilus* or *E.coli* had enhanced healing of intestinal anastomosis. The ex-germ free animals demonstrated a significantly slower rate of healing than either of these groups, suggesting that the healing effect may not be due to the presence of any bacteria but to specific types of bacteria within the intestinal environment. This animal study provides encouraging results for possible use in pouchitis treatment within humans.

The exact mechanism by which *Lactobacillus sp.* exerts protection against the development of inflammation is yet unknown. However, it is known that certain *Lactobacillus sp.* adhere to mucosal surfaces and sterically inhibit the attachment of aerobic gram-negative bacteria (Gill et al., 2000). In addition, *L.reuteri* has been shown to secrete inhibitory products that have antimicrobial activity against potential pathogens. *Lactobacillus sp.* also reduces pH in the colonic lumen, which may decrease the growth of pathogenic bacteria (Gill et al., 2000).

Some of these animal studies demonstrate a positive impact of probiotic treatment on attenuation of IBD, while others have not found a beneficial effect. This could be due to the inherent differences in strains of bacteria used or the types of animal models differ. Moreover, the time of initiation of probiotic treatment could also affect experimental outcomes. Regardless, these experiments have established an important link between dysbiosis and the development of CD as well as the ability of some strains of LAB to alter this dysbiosis.

1.4.4.2 Human Studies

Some of the earliest documented clinical use of probiotics for the treatment of IBD in a clinical setting occurred in the late 1980s. Interestingly, Borody et al., (1989) suggested that patients could benefit by having the infected resident bowel flora removed by gastrointestinal lavage and replaced with normal bowel bacteria from a healthy donor, thus resulting in the ‘crowding out’ of the remaining pathogens. They studied this treatment on 55 patients who were experiencing constipation, diarrhea, abdominal pain, UC or CD. Out of this study group, 20 individuals were deemed cured, 9 had slight alleviation of symptoms and 26 failed to show improvement. At the 12-month follow-up, several of the cured individuals still had no further symptoms. Similar experiences were observed by Bennet and Brinkman (1989) for the treatment of ulcerative colitis.

Although these two trials were not well-designed clinical trials, they do suggest that a normal intestinal microflora may confer resistance to colonization by pathogens and functions as an important constituent of the gut defense barrier. Moreover, reinforcing the hypothesis that dysbiosis is a major contributing factor in the etiology of IBD.

1.4.4.2.1 Crohn's Disease

In human clinical trials, the results are just as diverse, where factors such as time of administration and type of strain administered have a significant impact upon disease attenuation or progression. However, there are clinical trials that demonstrate a significant role of probiotics in the attenuation of disease.

Gupta et al. (2000) performed a 6 month open-label pilot evaluation of the efficacy of *Lactobacillus GG* in children with CD. Children with mildly to moderately active CD were given *Lactobacillus GG* (10^{10}colony forming units (CFU)) in enterocoated tablets twice a day for 6 months. They found that there was significant improvement in clinical activity 1 week after starting the treatment protocol; this improvement was sustained throughout the treatment period. In addition, intestinal permeability was found to have improved in a similar fashion.

The researchers concluded that *Lactobacillus GG* might improve gut barrier function and clinical status in children with mildly to moderately active, stable CD. Although, this trial only had a limited number of subjects (n=4), it provides preliminary evidence that a randomized, double blind, placebo controlled trial is warranted to assess the efficacy of *Lactobacillus* GG in CD.

Malchow, (1997) in a placebo controlled study, investigated whether *Escherichia coli* strain Nissle 1917 had a beneficial effect on restoration of the physiologic intestinal microflora in colonic inflammation by exogenous administration of a viable nonpathogenic bacterium. *E.coli* strain Nissle 1917 has no pathogenic potential aside from the ability to colonize within the intestine; furthermore it can inhibit the growth of enteropathogenic and uropathogenic *E. coli* and other enteric bacteria that may be involved in the pathogenesis of IBD. Prednisolone, which controls the inflammation and interrupts immunopathogenic mechanisms, was administered along with *E. coli*.

This study found promising results in terms of efficacy and tolerance in using nonpathogenic *E. coli* strain Nissle 1917 in maintaining remission in patients with colonic CD. Physicians as well as patients regarded tolerance to the probiotic treatment as good or very good. Within the *E.coli* group, 33.3% of the patients had a relapse during the 1-year treatment compared with 63.6% in the placebo group. Of the patients who had stopped taking prednisolone before the relapse, only 30% of the *E. coli* compared with 70% of the placebo patients experienced a relapse, however, this was not found to be significantly different. In addition, patients treated with prednisolone and *E.coli* had fewer relapses than did patients in the control group, and were able to stop the steroid treatment during the trial. The researchers concluded that the application of the physiologic bacteria reduced the risk for relapse and minimized the need for glucocorticoids. It is hypothesized that the *E.coli* probiotic preparation acts by producing microcines and by creating a competition for substrates, vitamins and growth factors with other gastrointestinal bacteria. This probiotic treatment was expected to have a long-term effect by suppressing enteropathogenic bacteria, thus maintaining remission (Malchow, 1997). However, this study did not perform microbiologic analysis to confirm whether there was a change in the gut flora of the individual patients nor did they indicate relapse rates after cessation of treatment.

Guslandi et al. (2000) studied the effect of co administration of probiotic yeast, *Sachharomyces boulardii* with mesalamine on the maintenance of remission in CD. Patients were given mesalamine (1 g, 3×/day) or mesalamine (1 g, 2×/day) plus a preparation of *Saccharomyces boulardii* (1g/day). These patients were then followed for 6 monthes. After six months, only 6.25 % of the patients receiving the probiotic treatment had a clinical relapse, whereas 37.5% of the group receiving only mesalamine had a clinical relapse. Furthermore, treatment with probiotics have been studied to maintain remission in surgically induced remission. Prantera et al., (2002) demonstrated that although patients remained in remission, remission rates were not different between patients given *L. casei* subsp. *rhamnosus* than the placebo control. Conversely, Campieri et al. (2000) demonstrated that VSL# 3 was just as effective as mesalamine at maintaining patients in remission.

A decrease in mucosal immunity and barrier function has also been implicated in the etiology of IBD. It is believed that alterations in the mucosal epithelial lining results in the inability of the immune system to mount an immune reaction or to mount an improper immune reaction, leading researchers to find ways to augment or alter immune activation. Furthermore, researchers have found that LAB have the ability to enhance natural and acquired immunity (Gill et al., 2000). Secretory IgA in mucus and body secretions protects the mucous membrane from invasion by microorganisms. Thus, Malin et al., (1996) studied the immune responses, particularly IgA production, in patients with CD after oral bacteriotherapy with *L.rhamnosus*. Despite the short duration of the treatment (10 days) and the small number of subjects (14 children with CD; 9 with juvenile arthritis; 7 controls), they demonstrated increased numbers of IgA-secreting cells, particularly for those within the Crohn's group. They concluded that *L. rhamnosus* strain *GG* has the potential to increase the gut IgA immune response and promote the gut immunological barrier in patients with CD (Malin et al., 1996).

Although there are various studies that demonstrate that probiotics would be beneficial in the treatment of CD, there are others that dispute this claim. However, until results from well designed randomized double-blind placebo controlled trials with large numbers of patients analyzed, can solid evidence of the efficacy of probiotics in CD be found. Until that time, the use of probiotic in CD treatment has not been validated but further research is warranted.

1.4.4.2.2 Ulcerative Colitis (UC)

Similar to CD, dysbiosis is implicated in the etiology of UC. Researchers have been studying the use of probiotics to manipulate the intestinal microflora in treatment and maintenance of disease symptoms in UC.

In a small clinical trial (Venturi et al., 1999), VSL#3, a probiotic preparation containing 300 billions/g of viable lyophilized bacteria of 4 strains of lactobacilli (*L.casei, L. plantarum, L.acidophilus and L.delbruekii* subsp. *bulgaricus),* 3 strains of bifidobacteria (*B. longum, B. Breve, and B. Infantis)*, and 1 strain of *Streptococcus* *salivarius* subsp.*thermophilus,* was tested as a maintenance treatment in UC patients allergic or intolerant to sulphasalazine or mesalazine. This particular preparation has two important characteristics, which may prove advantageous over other probiotic preparations: a high bacterial concentration and the presence of a mixture of different bacterial species with potential synergistic reactions to enhance suppression of potential pathogens. In addition, it is well known, that each probiotic strain possess very different and specialized metabolic activity, such that claims made for one strain of an organism cannot necessarily be applied to another. Thus, theoretically, a mixture of a large number of probiotic strains may be the most effective.

In this particular study, twenty patients received 6g/day of VSL#3 for 12 months. Microbiological analysis showed a significant increase fecal in concentration of lactobacilli, bifidobacteria and *S. salivarius* subsp. *thermophilus*, which was evident after 10 days and persisted throughout the treatment period; no significant changes were observed in concentrations of other bacterial species. Fecal pH was significantly reduced by the treatment, and most patients (75%) remained in remission. However, the sample size in this study is fairly limited, decreasing statistical and extrapolation power.

In a larger study with a larger sample size, Kruis et al., (1997), compared the effects of probiotic treatment with an oral preparation of non-pathogenic *E.coli* as compared to the standard maintenance treatment of UC with mesalazine. One hundred and twenty patients with inactive UC were included in a double-blind, double dummy study comparing mesalazine 500 mg three times a day to an oral preparation of viable *E.coli* strain Nissle (Serotype O6:K5:H1) for 12 weeks. Tolerability for the treatment was excellent and did not differ between groups. They found that relapse free time was the same between both treatment groups. The relapse rates were 11.3% for mesalazine and 16.0% for *E.coli.* They concluded that probiotic treatment appears to offer another option for maintenance therapy of UC.

Although there was no significant difference found between the treatment groups in the study done by Kruis et al. (1997), follow – up was only for a short period of 12 weeks. Rembacken (1999) performed a double-dummy, randomized study to determine whether the induction of a lasting change in the colonic flora by pretreatment with oral gentamicin, followed by seeding with the non-pathogenic *E.coli*, is equivalent to gentamicin and mesalazine in inducing remission of active UC or preventing relapses over a 12 month period.

A total of 116 patients with an exacerbation of UC took part in this study. Individuals were randomized to either receive mesalazine 800 mg three times daily or a non-pathogenic strain of *E.coli* (serotype O6:K5:H1) Nissle 1917 at a dose of two capsules twice daily (2.5×10^{10} viable bacteria per capsule). All patients were also given a 1 week course of oral gentamicin 80 mg three times a day, at entry into the study, to suppress their native *E. coli* flora. The number of patients who achieved remission was 75% in the mesalazine group and 68% in the *E.coli group.* During the 1 year follow up, 73% and 67% relapsed in the mesalazine and the *E.coli* group respectively.

Although the results from this study are comparable to those of Kruis and colleagues (1997), the experimental design of this study is more powerful. It is known that the risk of relapse is the lowest when patients are recruited into trials during a long period of remission and the highest when they are recruited soon after an exacerbation. Neither Kruis nor Venturi (1999) controlled for this variable, so to reduce this source of bias the Rembacken (1999) study group recruited patients during the active stage of the disease and then followed them up prospectively into remission and then relapse. This method aids to standardize the population.

Rembacken (1999) suggests several possible mechanisms by which the non-pathogenic *E.coli* strain (Nissle 1917) may be having an effect. First, it may be blocking receptors in the intestinal mucosa and preventing adhesive pathogenic bacteria from becoming established. Second, the *E.coli* strain used had antagonistic activity against a variety of pathogenic and non-pathogenic enterobacteria, probably due to the production of bacteriocins and microcines, and may have eliminated other pathogenic bacterial strains. Third, the growth and metabolic activity of *E.coli* may have caused changes in the pH or chemical composition of the colonic lumen that are unfavourable to bacteria involved in the pathogenesis of UC or that promote repair and nourishment of the mucosa.

1.4.4.2.3 Pouchitis: Human Clinical Trials

In individuals with severe colitis, symptom alleviation is often achieved only through surgical resection and anastomosis. Although there is alleviation of colitis symptoms, other complications may arise; pouchitis is a common side effect. Pouchitis occurs when the ileal pouch is overgrown with bacteria resulting in inflammation. Thus modulation of the flora may also be of some therapeutic benefit in individuals with pouchitis.

Efficacy of the oral probiotic preparation used by Gionchetti et al., (2000), VSL#3 in the UC study, was then compared with placebo in the maintenance treatment of chronic relapsing pouchitis. Forty patients who obtained clinical and endoscopic remission after 1 month of combined antibiotic treatment (rifaximin 1g bid plus ciprofloxacin 500 mg bid) were randomized to receive either VSL#3 6g/day or placebo for 9 months.

All 20 patients on placebo had a relapse (8 within 2 months, 7 within 3 months and the remaining 5 within 4 months); in contrast, 17 of the 20 patients treated with VSL#3 remained in remission after 9 months, however, all 17 patients had a relapse within 4 months after the active treatment was stopped suggesting the need to continuously sustain the microbial treatment. No side effects were registered. Faecal concentration of lactobacilli, bifidobacteria and *S. salivarius subsp. thermophilus* significantly increased within 1 month of starting VSL#3 treatment and remained stable throughout the study. With regard to the mechanism of action of VSL#3, in the patients with pouchitis who received this preparation there was a significant increase of interleukin (IL)-10 tissue levels, while tumour necrosis factor-α, IL-1β and interferon gamma concentrations were not modified. Mimura et al. (2002) observed nearly identical results in a double blind, placebo controlled study. The only differences between the two experiments is that Mimura administered the same concentration of VSL#3 in a single dose and their treatment period was for 1 year instead of 9 months.

Furthermore, VSL# 3 was studied by Gionchetti et al., (2003) on the prevention of pouchitis immediately after surgical resection. In a double blind randomized control trial, individuals either received VSL#3 (9×10^{11} bacteria/day) or a placebo. Patients were maintained on this therapy for twelve months commencing treatment 1 week post operation. In the treatment group, nearly 90% were pouchitis free whereas 60% of the placebo group had relapsed 1 year after surgical resection.

However, there has been limited success in using probiotics to treat active pouchitis. In clinical trials using a single probiotic strain (*L. rhamnosus* GG) there was no difference between pretreatment and post treatment disease activity (Kuisma et al., 2003). Nor was there a change in disease parameters, when subjects were given 500 g/day of the dairy product Cultura (which contains *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12) for 4 weeks (Laake et al., 2003). Interestingly, in both of these studies, although they did not observe a change in disease parameters, there was a significant increase in the numbers of lactobacilli present in the gut. This illustrates the importance of selecting strains with specific characteristics that are beneficial to the disease and that there are indeed significant differences in activity between different strains.

1.4.5 Treatment of IBD with Fermented Milk and Milk Protein

The majority of the studies performed have used probiotics in capsules and lyophilized forms. However, a small number of studies have used milks fermented with probiotics as the carrier for treatment of UC and pouchitis

Ishikawa et al. (2002) studied the effect of bifidobacterium fermented milk (BFM) on maintaining remission in patients with UC. Patients were randomized into either the BFM supplemented treatment group or the standard treatment group (salazosulfapyridin, mesalazine and steroids). For patients in the BFM group, they were given 100 mL/day of BFM for one year. At the end of the one-year treatment period, 3 out of 11 of the BFM group had exacerbation of symptoms; whereas the control group had 9 out the 10 patients relapse. This was statistically significant. In addition, patients on the BFM group reported less standard treatment associated side effects. Furthermore, in a randomized placebo controlled trial, patients with active colitis were given a BFM supplement (100 mL/day) in addition to standard treatment (mesalazine and salazosulphapyridine) or just given standard treatment. BFM significantly decreased the clinical disease activity as compared to placebo control. In addition, in the BFM group 70% of the patients responded to the treatment and 40% attained clinical remission. In the placebo control group, only 33% of the patients responded and 33% were able to attain clinical remission (Kato et al., 2004).

In patients who required surgical resection to control symptoms of UC fermented milk cultures have been tested. As previously described in a study performed by Laake et al. (2003) patients who had undergone resection and who were given 500 mL of Cultura, did not have any change in clinical parameters. In fact these researchers suggested the probiotics acted only superficially. However, treatment with Cultura was only maintained for 4 weeks. Perhaps the treatment period was too short to detect a significant effect. In a further study with BFM, measuring a larger array of clinical parameters Laake et al., (2005) found that the same treatment regimen significantly improved disease status. Patients receiving this treatment had significantly less, involuntary defecation, leakage, abdominal cramps, and the need for napkins, faecal number and frequency and mucus and the urge to evacuate stools with Cultura treatment.

The use of fermented milk is an interesting treatment modality. In addition, to supplying the colon with probiotic bacteria there is also the presence of bioactive peptides that are natively present within milk. As previously discussed, through bacterial and gastrointestinal enzymatic digestion or through lactic acid degradation during fermentation, protein within milk is digested into bioactive peptides. Indeed, milk proteins have been shown to possess immunomodulatory, antinflammatory, anticancer and antimicrobial activity. However, studies have focus more on the role of the probiotic used to ferment the milk in treatment of IBD than on the proteins/peptides liberated from milk during fermentation and processing. In spite of this, due to the physiological functions attributed to milk proteins, it would be logical to assume that the use of milk proteins would also be a feasible treatment modality in IBD. Unfortunately, this area is not very well studied. Only a few studies, using elemental diets allude to the role of milk proteins in the attenuation of IBD (Donnet-Hughes et al., 2000).

Furthermore, since IBD is thought to be a disease of dysbiosis, theoretically, due to the iron-limiting antimicrobial associated activities of lactoferrin, this milk protein could be used in conjunction with other treatment to eradicate pathogenic bacteria.

Furthermore, in humans, Jones et al., (2004) reported the benefits of a casein based enteral diet in controlling disease parameters in a 40 year old female with Crohn's disease. After being treated for 5 days with the casein-based diet, the patient no longer required opiate and non-opiate analgesics to control pain. Jones et al., suggested that this effect could be due to bioactive proteins, such as transforming growth factor, naturally present in milk that are able to down regulate the inflammatory response and thus induce remission. Others have reported similar findings, however these were in paediatric patients (Donnet-Hughes et al., 2000).

In another study, the effect of oral polymeric diet (CT3211; Nestle, Vevey, Switzerland) on mild to severe active CD in children was measured (Fell et al., 2000). CT3211 is a casein-based polymeric diet that is rich in transforming growth factor β2. Each patient was prescribed CT3211 as the sole source of nutrition for 8 weeks concurrent with medical treatment; volumes were calculated according to caloric requirements. After eight weeks of treatment 23 patients were in clinical remission. Furthermore, there were also endoscopically measured improvements; TNFα serum levels were significantly lower after treatment, as well as other immunological parameters. The researchers suggest that the polymeric diet reduced mucosal inflammation through modulating inflammatory cytokines such as TNF-α (Fell et al., 2000). A similar study using a different casein based diet (ACD004, Nestle) did not showed similar improvements in disease parameters (Afzal et al., 2004). However, in the study by Afzal et al. (2004) the patients were administered a questionnaire (IMPACT II) to determine quality of life measures. After treatment with the casein based diet as the sole source of nutrition, there was an overall improvement in quality of life scores such as emotional functioning, social functioning and body image for all children enrolled in the study (Afzal et al., 2004). However, the precise mechanism with which casein based enteral feeds improves IBD in children remains to be elucidated

Indeed, in studies of tissue collected from patients with inflammatory bowel disease treatment with elemental diets based on casein or whey was examined. In tissues isolated from patients with CD, who were treated with casein based elemental diet there was a substantial increase in antinflammatory cytokines, whereas, tissues from patients with UC were not affected. This suggests a role for casein as a potential therapeutic agent in CD (Meister et al., 2002). In trinitrobenzenesulfonic acid-induced colitis in rats, the administration of bovine glycomacropeptide dose-dependently decreased the degree of inflammation inflammation, weight loss, anorexia, and colonic damage and also decreased inducible nitric oxide synthase mRNA levels (Daddaoua et al., 2005).

However, there are very few studies done on the effect of milk proteins on IBD. The majority of the studies are focused upon probiotic modulation of disease state. Information regarding milk proteins and peptides present in individually or in fermented milk on the etiology of IBD is very limited and in its infancy. However, there is tremendous potential for the use of milk proteins as an adjuvant to treatment in IBD.

1.5 CONCLUSION

Due to the increasing prevalence and development of antibiotic resistance, the time has clearly come to increase the exploration of novel methods of treating IBD. Probiotics represent a potentially significant therapeutic advance in treatment of IBD, particularly in pouchitis. Probiotics are advantageous to the host health in a number of ways. They offer a dietary means to support the balance of the intestinal flora or in many disease states, a method to manipulate the intestinal microbiota. They may be used to counteract local immunological dysfunction, to stabilize the intestinal mucosal barrier function, to prevent infectious succession of pathogenic microorganisms and to influence intestinal metabolism.

The initiation and perpetuation of chronic IBD seems to be intimately associated with the luminal bacterial flora. CD occurs at sites with the highest concentration of anaerobic bacteria and is, to a certain extent, treatable with antibiotics and bowel rest. Several studies, using animal models susceptible to experimental colitis, show conflicting effects of probiotics in the treatment of chronic IBD. In spite of this, pilot trials including patients with pouchitis, UC and CD have found some promising results that probiotics can alter the course of the disease. However, many of these studies were not based well-designed experiments nor did they control for variables that could bias the results, thus limiting our ability to extrapolate results.

Furthermore, the study of milk proteins in the treatment of IBD is in its infancy. There are only a few reports on the use of whey or casein based elemental diets in the successful management of IBD. However, considering the many biological functions attributed to milk proteins and peptides, they represent a significant treatment modality. Further research is required to determine the bioactve functions of milk proteins *in vitro*, then selection of the ideal protein mixture with treatment characteristics for IBD will then be possible.

Cancer is a disease of rapidly increasing prevalence. There is increasing evidence indicating the possible role of probiotics in controlling cancer growth. However, human and animals studies have demonstrated many contradictory findings. The type of probiotic strain, time of administration, and the final bacterial load among other things effect the experimental outcome. Furthermore, each bacterial strain is functionally very different from another similar strain, thus limiting extrapolation of functionality to other strains. Moreover, the majority of the strains tested are of human origin, and thus far have met with only some success. However, as previously discussed, studies are beginning to demonstrate the therapeutic value of LAB isolated from many food items. Dairy isolated strains represent a significant source of possible new strains of probiotic LAB. Many of these strains are currently present in commercially available dairy products, or would not pose a significant technological problem to incorporate into a dairy product.

There is increasing evidence that in fermented products LAB bacteria are not the only bioactive agents. Researchers have suggested the presence of other substances created/liberated during the course of fermentation that also have bioactive properties. As previously discussed, fermentation and processing liberate bioactive peptides/proteins from the native milk structure; thus the consumption of fermented dairy products would not only contain probiotic LAB but bioactive milk proteins/peptides. This represents a significant source of highly digestible bioactive proteins and peptides.

Nevertheless, any postulated benefit from consumption of probiotics or milk proteins should be accepted as fact only after extensive testing. There are many unresolved issues that can be answered only by well-designed and well-controlled clinical trials that are preceded by rigorous *in vitro* experimentation that elucidate spectrums of activity and to identify other strains with probiotic qualities. Furthermore, the majority of studies demonstrate that probiotics and bioactive proteins are able to prevent or inhibit the development of diseases such as colon cancer and IBD, however, there is a strong lack of information in regards to the understanding of the mechanisms by which these bioactive components exert these effects. The understanding of these mechanisms will allow the development of definitive criteria for the appropriate selection of probiotic strains and milk proteins and peptides with precise spectrum of activities to treat specific disease. In addition, the understanding of mechanisms of action will allow the development of cocktails of strains with synergistic activities in the treatment of disease.

1.6 RESEARCH OBJECTIVES

Functional foods and nutraceuticals have become a major opportunity as possible prevention/treatment modalities for many diseases. Increased research is being driven by many factors. Consumers are becoming more health conscious and are demanding food products that are not only tasty, convenient and nutritious, but provide some sort of healthful benefit. Furthermore, there is growing understanding between the etiology of many diseases and dietary practices. Thus, there are a growing number of products available on the market with unsubstantiated health claims. Dairy products represent a major portion of functional foods within the Japanese, European and Australian markets, whereas, North American markets are only beginning to grow. However, growth is at a tremendously rapid rate.

As discussed before, there is tremendous potential for the use of probiotics and milk-derived probiotics as functional ingredients in food. Clinical trials and animal studies have demonstrated a role of probiotics and milk proteins in the treatment of various cancers and IBD. Clinical trials and animal studies give us both indirect and direct efficacy of probiotics, however, due to the inherent complexities of these system confounding variables make it difficult to determine exactly how functional ingredients alter physiological functions. Although not as sophisticated as animal and human studies, *in vitro* experiments provide a controlled system with which to observe physiologic reactions and thus mechanisms of action. Thus, the overall objective of this research is to identify new functional ingredients derived from dairy LAB and proteins that can be used as dietary adjuvants in the treatment of gastrointestinal disorders. The following are the objectives for each individual experiment.

Objective 1. Cancer is a complex process, involving derailment of many of the body's protective mechanisms. The initial step in the development of cancer is mutation. Mutations occur at relatively high rates within the body, however, DNA repair mechanisms are constantly surveying the body for such mutations and initiate corrective actions. However, some mutations are missed by these repair systems and may eventually develop into cancer. Thus the isolation of antimutagenic compounds naturally present in food has immense therapeutic/preventative implications. The objective of this study is to investigate the antimutagenic potential of *L. casei* ADA 03 (live cells, cell wall peptidoglycan, fermented milks and fermented milk supernatant) and dairy derived proteins/peptides (β-casomorphin-7, β-lactoglobulin, casein hydrolysate, sodium caseinate and glutathione) against 2 aminofluorene induced mutation of *Salmonella typhimurium* TA 98, in the Ames Salmonella reverse mutation assay.

Objective 2. Adhesion is the mechanism that bacteria use to transiently colonize the gut, preventing rapid removal by peristaltic contractions. Furthermore, adhesion increases the residency time of LAB within the gut, to allow maximum time for probiotic effects. Thus, adhesion to the gut is one of the primary selection criteria for a strain of LAB to become a probiotic. The objective of this study was to investigate the adhesive qualities of dairy derived LAB (*Lactobacillus acidophilus* MR 100, *Lactobacillus bulgaricus* MR 110, *Lactobacillus acidophilus* NRC 13017, *Lactobacillus acidophilus* NRC 13019, *Lactobacillus casei* ADA 03, *Lactobacillus casei* ADA 05, *Lactobacillus casei* subsp. *casei* CSCC 2601 and Yogurt Culture YC085) to human colon cancer cell line HT29.

Objective 3. Nitric Oxide (NO) and inducible nitric oxide synthase functioning is tightly regulated in the body and a powerful component of immune function. However, in some disease states, this tightly regulated system is derailed. For this reason, it seems logical that NO is a therapeutic target in disease resolution. Macrophages are key immune modulators against infection, inflammation and tumour development. The production of NO by macrophages is an integral component of the body's antipathogenic and tumouridical. Dairy derived LAB and bioactive peptides have been found to have anticancer and anti-inflammatory properties in both cancer and IBD (Brady et al., 2000; Varcoe et al., 2003; Fedorak et al., 2004). The antitumour/anti-IBD activities have generally been ascribed to binding of mutagenic/carcinogenic compounds, inhibiting noxious metabolite formation, protection against DNA demethylation (Donnet-Hughes et al., 2000; McIntosh et al., 1998a), or altering deleterious microflora and their respective enzymes (Kulkarni and Reddy, 1994). Recently, the focus has shifted to immunostimulatory or immunomodulatory effects of dairy derived LAB and proteins. The objective of this study was to identify whether several dairy derived LAB and milk proteins can have immunomodulatory potential through altering NO secretion by *Salmonella typhimurium* LPS stimulated or unstimulated macrophages. Furthermore, we wanted to determine which components exert the effects.

Objective 4. It is well known that there are two distinguishable mechanisms of cellular death; necrosis or apoptosis. Necrosis is classified as "accidental death" that often occurs when the cell is exposed to some physical or chemical treatment while apoptosis is a natural cell death process by which old or unusable cells are removed. Cytotoxicity does not define a specific cellular death mechanism, it is simply the cell killing property of a chemical compound (such as a food, cosmetic or pharmaceutical) or a mediator cell (such as a cytotoxic T cell), independent from the mechanisms of death (Roche Applied Science, Apoptosis, Cell Death and Cell Proliferation Manual). The purpose of the present study is to determine whether dairy derived LAB and proteins/peptides have cytotoxic activity against HT29 human colon cancer cells.

Objective 5. Many defective or dangerous cells are able to trick the apoptotic mechanisms within the body. Recent research has shown the possible use of probiotics and milk derived proteins to treat colon cancer (Di Marzio et al., 2001; Marchetti et al., 1997; Heerdt et al., 1997; Luhrs et al., 2002; Jan et al., 2002). In cancer research, there is much evidence indicating the role of apoptosis in cancer development and malignancy (Wynford-Thomas, 1996); thus leading to research on apoptosis inhibiting or stimulating agents. The purpose of this work is to assess the direct effect of several strains of dairy derived LAB and proteins on the apoptotic and necrotic activity against HT29 human colon cancer cells.

Objective 6. Tumour necrosis factor alpha (TNF α) and interleukin 6 (IL 6) are pleiotropic cytokines with powerful and wide-ranging effects upon the immune system. In IBD unrestrained activation of both of these inflammatory cytokines seems to play a pivotal role in the pathogenesis of altered mucosal immune function (Van Deventer, 1997; Atreya and Neurath, 2005). Research on modulation of IL 6 is relatively in its infancy. Only recently has there been evidence suggesting IL 6 has been positively correlated with the pathogenesis of colon cancer. Schneider et al (2000) demonstrated that IL 6 stimulated the growth of human colon cancer cells *in vitro;* several carcinoma cell lines secrete IL-6 (Basolo et al., 1996; van Meir et al., 1990; Watson et al., 1990). Moreover, IL 6 serum concentration in patients with colorectal cancer are dramatically elevated and positively correlated with progression of disease and mortality (Chung and Chang, 2003). Indeed, in patients with CD serum IL 6 levels are also elevated (Holub et al., 1998) and have been positively correlated with inflammation (Reinisch et al., 1999). Furthermore, the administration of anti-interleukin 6 monoclonal antibodies in murine models of experimental colitis prevented disease associated wasting and inhibited the development of lesions (Ito, 2003).

TNF is considered to be a key inflammatory cytokine involved in the dysregulation of IBD and cellular growth in cancer. TNF is a pivotal player in cellular proliferation, differentiation and apoptosis, as well as inducing other cytokines and immunoregulatory mediators (Papadakis and Targan, 2000). In CD, TNF α up regulates many inflammatory mediators, activates neutrophils, macrophages and stimulates B cells. The inhibition of TNF α production in IBD has promising therapeutic benefits in the treatment of CD (Lakatos, 2000). Indeed, the inhibition of *Helicobacter hepaticus* induced IBD, was achieved with the administration of *Lactobacillus casei,* which down regulated TNFα secretion (Pena et al., 2005).

The objective of this study is to isolate dairy derived milk proteins/peptides and LAB that have immunomodulatory effect on TNFα and IL-6 production. In addition, we wanted to determine if a cocktail of the eight strains tested has augmented effects on TNFα/IL-6 production, through synergy between the several bacteria. The effects could possibly then be applied into a dairy based functional food product with immune altering capabilities both for prevention in healthy individuals or as an immunoadjuvant to treatment in gastrointestinal disorders.

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Table 1.1. Major Proteins in Milk: Biological Functions and Concentration

		Concentration (g/L)		
Source	**Protein**	**Cow**	**Human**	**Biological Function(s)**
WHEY[a]	β-lactoglobulin	3.2		Retinol carrier, binding fatty acids, possible antioxidant; anticarcinogenic
	α-lactalbumin	1.2	1.9	Lactose synthesis in mammary gland, Ca carrier, immunomodulation, anticarcinogenic
	Immunoglobulins	0.7	1.3	Immune protection
	Lactoferrin	0.1	1.5	Antimicrobial, antioxidant, immunomodulation, iron absorption, antitumour
	Lactoperoxidase	0.03		Antimicrobial
	Serum albumin	0.4	0.4	Source of essential amino acids
	Glycomacropeptides[d]	1.2		Antiviral, bifidogenic; digestive function
CASEIN[b]	α-Casein	13.0	2.7	Effect on digestion
	β-casein	9.3		Opiate effect, antimicrobial, immunomodulation
	κ-casein	3.3		Immunomodulation
	β-casomorphins[c]			Effect on digestion, Opiate effect

Adapted from Marshall, 2004; Schlimme and Meisel, 1995; Severin and Wenshui, 2005; Danone World Newsletter, 1998; McIntosh et al., 1998b

a. Total Whey concentration – cow milk (6.3 g/L); human milk (67.3 g/L)
b. Total Casein concentration – cow milk (26.0 g/L); human milk (2.7 g/L)
c. Derived from β-casein
d. Glycomacropeptide derived from the casein fraction, however, it is included here as it appears in the whey after cheese making (McIntosh et al., 1998b)

Table 1.2 Bioactive Peptides Derived from Milk

Original Milk Protein	Bioactive Peptide	Bioactive Properties
α-casein	α-casomorphin	Opiate activity
	Exorphin α casein	Opiate activity
	Casokinin	Antihypertensive
β-casein	β-casomorphin	Opiate activity
	Casokinin	Immunomodulatory and antihypertensive activity
	Caseinophosphopeptide	Effect on mineral status
κ-casein	Casokinin	Opiate antagonist
	Casoplatellins	Antithrombotic activity
α-Lactalbumin	Fragments 50-53	Opiate activity
β-lactoglobulin	β-lactorphins	Opiate and antihypertensive activity
Lactoferrin	Lactoferricin	Antimicrobial
Serum Albumin	Serorphin	Opioid agonist

(Danone World News Letter, 1998; McIntosh et al., 1998b)

CHAPTER 2[*]

Antimutagenic Activity of *Lactobacillus casei* ADA 03 and Milk Derived Bioactive Peptides using the Ames *Salmonella Typhimurium* TA98 Reverse Mutation Assay

2.1 INTRODUCTION

The human gastrointestinal tract is comprised of approximately 400 bacterial species; *Lactobacillus* and *Bifidobacterium* species are dominant members (Naidu et al., 1999). Lactic acid bacteria (LAB) are conventionally referred to as probiotics, which are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being of the host (Salminen et al., 1999)

LAB have played a tremendous role in maintenance of the food supply. Traditionally LAB have been exploited for their role in food fermentation, preservation and contributions to the organoleptic profiles in food. However, recently LAB and their products of fermentation have gained attention as functional food ingredients. Functional foods are bioactive substances found naturally in food that may modulate the risk of disease development through its effects on physiological or other functional processes within the body. Many beneficial physiological effects have been attributed to the consumption of LAB and products of fermentation. LAB have been shown to enhance immune function through modulation of various cytokines and immune potentiating chemokines (Dotan and Rachmilewitz, 2005; Gardiner et al., 2002), increased resistance to infections (Savadogo et al., 2004) and antitumour activities (Aso et al., 1995). Moreover, the process of fermentation by LAB has been shown to liberate bioactive compounds with potent bioactive properties (Gobbetti et al., 2002; Ruas-Madiedo et al., 2002).

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Animal studies have demonstrated that consumption of LAB can inhibit cancer development (Macleod et al., 1990) and modulate biomarkers of carcinogenesis, such as ornithine decarboxylase activity, affect the activation state of ras protooncogenes (Singh et al., 1997) or stimulate the immune system (Perdigon et al., 1998). Interestingly, Varcoe et al. (2003) showed that co-administration of carcinogen and LAB did not have any effect on colon cancer development, whereas prophylactic administration resulted in attenuation of colon hyperplasia in rats. Furthermore, epidemiological evidence suggests a strong negative correlation between the development of certain cancers and the consumption of fermented dairy products. Van't Veer et al. (1989) found that in women in the Netherlands that had a higher intake of fermented milk products, there was a statistically significant decrease in the risk of developing breast cancer. Moreover, in a large prospective study of 110 792 patients (age 40-79 years), yogurt intake was inversely associated with rectal cancer mortality (Kojima et al., 2004). Thus there is epidemiological evidence indicating that the consumption of fermented dairy products may have an effect upon modulation of certain disease states.

Cancer is a complex process, involving derailment of many of the body's protective mechanisms. The initial step in the development of cancer is mutation. Mutations occur at relatively high rates within the body, however, DNA repair mechanisms are in constant surveillance of the body for such mutations. Upon detection of a DNA mutation, these repair systems initiate corrective actions. However, some mutations are missed by these repair systems and may eventually develop into cancer (World Cancer Research Fund, 1997). Thus the isolation of antimutagenic compounds naturally present in food has immense therapeutic/preventative implications.

This study was undertaken to investigate the antimutagenic potential of *L. casei* ADA 03 (Live cells, peptidoglycan, fermented milks and fermented milk supernatant) and dairy derived proteins/peptides (β-casomorphin-7, β-lactoglobulin, casein hydrolysate and sodium caseinate) against 2 aminofluorene (2AF) induced mutation of *Salmonella typhimurium* TA 98, in the Ames Salmonella reverse mutation assay.

2.2 MATERIALS AND METHODS

2.2.1 *Salmonella typhimurium* TA98 Propagation and Growth

The tester strains of *Salmonella typhimurium* (TA98) were kindly provided by Prairie Biological Research (Edmonton, Alberta, Canada). *Salmonella typhimurium* TA98 was originally derived from *Salmonella typhimurium,* strain LT2. This particular strain contains a hisD3052 mutation, which makes it sensitive to frameshift mutations.

Fresh cultures of *Salmonella typhimurium* TA98, were grown overnight (10-12 hours) in Oxoid Nutrient Broth no. 2 (Difco, Detroit, MI, USA) at 37°C in a shaking water bath up to the late exponential or early stationary phase of growth (approximately 10^9 cells/mL) and used immediately for antimutagenicity testing. Aliquots of stock culture were stored in dimethylsulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA) at –70 °C until required for antimutagenicity testing.

2.2.2 Probiotic LAB Propagation and Growth

L. casei ADA 03 used for antimutation testing was obtained from stock cultures available in our laboratory. An aliquot of bacterial culture was stored at –80 °C in MRS broth (Difco) supplemented with 20% (v/v) sterile reagent grade glycerol as a cryoprotective reagent (Sigma Aldrich) until required for use in experiments.

A 1% (v/v) inoculum of overnight culture of *Lactobacillus casei* ADA 03 from frozen stock cultures was grown in lactobacilli MRS broth (37 °C for 24 hours) and used immediately for testing. Experimental cultures were subcultured twice to minimize the amount of cryoprotective agent in the final incubation mixture.

2.2.3 *L.casei* ADA03 Peptidoglycan Isolation and Purification

Peptidoglycan was isolated from *L.casei* ADA 03 according to the methods reported by De Ambrosini et al., (1996). Briefly, resuspended cells of *L.casei* ADA 03 in PBS were subjected to mechanical disruption using a French Press. Broken cells were centrifuged (10 000 g) for 10 minutes and the pellet obtained was suspended in 4% boiling SDS (Sigma Aldrich) in order to dissolve the cell wall components. After 18 hours of incubation, the suspension was centrifuged at 20, 000 g for 20 minutes, and washed with PBS to remove SDS. The pellet was then treated with RNase and DNase to remove nucleotides, followed by washing with PBS. The pellet suspended in PBS was then treated with trypsin to digest any cell wall associated proteins. This preparation of cells was then washed with PBS and treated with 2% SDS for 4 hours to remove all proteins. Then the sample was washed thoroughly with PBS and deionized water (Milli Q, Millipore, Billerica, MA, USA). This fraction was considered as the whole cell wall preparation.

The insoluble cell wall precipitate was then treated with 10% trichloroacetic acid (Sigma Aldrich) for overnight period to remove teichoic acid from in the cell wall. The suspension was centrifuged and the pellet was washed thoroughly with PBS and MilliQ water. This pellet was taken as peptidoglycan and used for experimental testing suspended in sterile DMSO (dimethylsulfoxide).

2.2.4 *L.casei* ADA 03 Fermented Milk and Fermented Milk Supernatant

An aliquot of *L.casei* ADA 03 from stock cultures at −80 °C was grown overnight in MRS broth (Difco) at 37 °C. A 1% inoculum of the overnight culture was used to subcultured in 12% (v/v) skim milk (Parmalat, Canada) supplemented with 1% glucose (Sigma Aldrich) and 1% yeast extract (Sigma Aldrich); this was incubated for 24 hours at 37 °C. Skim milk cultures (10 mL) were centrifuged at 10 000 g for 10 minutes. The supernatants were collected and passed through a 0.2 μm pore size sterile filter (Acrodisc, Gelman Sciences, Ann Arbor, MO, USA). Aliquots of the supernatants were stored at −20°C until used for antimutagenicity testing.

For the 24 hour and 7 day fermented milk samples, *L. casei* ADA 03 was grown as described above except that the samples were grown for 24 hours or 7 days and used as fermented milk samples for antimutation testing.

2.2.5 Milk Protein/Peptide Preparation

Sodium caseinate, β-lactoglobulin, casein hydrolysate and β-casomorphin-7 were all purchased from Sigma Aldrich. The following concentrations were used for each protein/peptide fraction: sodium caseinate: 75, 37.5 and 18.75 mg/mL; β-lactoglobulin: 120, 90, 60, 30, and 15 mg/mL; casein hydrolysate: 40, 20 and 10 mg/mL and β-casomorphin-7: 125, 62.5 and 31.25 μg/mL. Each protein/peptide solution was filtered with a 0.25 μm syringe filter (Acrodisc, Gelman Sciences). The sterile solutions were made immediately prior to antimutagenicity testing.

2.2.6 Ames Salmonella Reverse Mutation Assay

The Ames Reverse Mutation Assay was used to determine the antimutagenic activity of *L.casei* ADA 03 (Live, 24 hour and 7 Day fermented milk, 24 hour fermented milk supernatant and peptidoglycan) and milk derived protein or peptide fractions against 2AF induced mutations. The assay was performed according to methods developed by Bruce N. Ames et al., (1975) with slight modifications.

The Ames test was originally developed to evaluate mutagenic properties of various suspected environmental mutagens. The test uses various strains of bacteria that due to a mutation are amino acid dependent, thus these organisms require an external source of histidine, without this the organism can not grow to form colonies. Colony growth is reestablished, when a test chemical causes a reversion in this mutation, allowing the production of histidine to resume. For our experimental purposes, in addition to adding a known mutagen a potential antimutagenic compound will be added in attempt to block reversion.

Briefly, we followed the plate incorporation method. Overnight cultures of *Salmonella typhimurium* TA98 (100 μL), 100 μL of the known mutagen 2 AF (Sigma Aldrich; 10 μg/mL dissolved in DMSO), 500 μL of rat liver S9 (Aroclor ™; prepared from adult Sprague Dawley rats; supplemented with glucose-6-phosphate, NADP-Na_2 to a final protein concentration of 2 mg/mL within the incubation mixture; BD biosciences, Mississauga, ON, Canada) and 100 uL of each protein/peptide solution or bacteria are added to molten top agar (Sigma Aldrich; 37 °C; 0.6% bactoagar, 0.5% NaCl plus 10 mL of 0.5 mM histidine/biotin and 0.5mM tryptophan solution/100 mL of top agar). The incubation mixture was immediately vortexed and poured onto the surface of Vogel-Bonner medium E (minimal medium; Sigma Aldrich) plates. The plates were then incubated for 48-72 hours at 37°C. Colonies were counted with an automatic colony counter. Each test was performed a minimum of three times in triplicate.

2.2.7 Statistical Analysis

Statistical Analysis was carried out in the Statistica program (Statistica 6.0). The data was analyzed through analysis of variance, Tukey's test, correlation of analysis and regression analysis.

2.3 RESULTS AND DISCUSSION

The prevalence of cancer within North American and Western European countries is on the rise. The etiology of many of the cancers in westernized nations has been attributed to dietary and lifestyle factors. In fact 75% of cancer has been attributed to dietary and lifestyle factors, with 50% of these linked directly to dietary factors. In particular, a strong correlation has been found between dietary factors and colorectal cancer (World Cancer Research Fund, 1997).

Cancer is a multistep process, in which each step provides opportunities to employ and exert antineoplastic agents and thus modulate the cancer process. Mutation is the initial step involved in the cancer process. Mutations occur in cells quite frequently; the majority of these are repaired by the body's DNA repair systems. However, some mutations are not repaired or missed by the repair systems resulting in a potentiation and accumulation of genetic defects that can ultimately culminate in cancer. Augmentation of DNA repair systems through modulation of mutagenic events with antimutagenic compounds found naturally in food is an attractive cancer therapeutic or preventative option.

It has been reported that diets high in grains, dietary fiber and green leafy vegetables can reduce the risk of development of various types of cancer (World Cancer Research Fund, 1997). Recently, dairy products and LAB used to ferment dairy products have become the focus of functional food and anticancer research. Epidemiological evidence indicates that increasing yogurt and dairy product intake is negatively associated with cancer development (van't Veer et al., 1989; Kojima et al., 2004). Furthermore, *in vivo* and *in vitro* studies have established that yogurt or LAB have antitumour activity against various tumours (Arimochi et al., 1997; Aso et al., 1995; Eliassen et al., 2002). However, the mechanism through which dairy derived LAB and proteins/peptides exert antitumour activities are not well known. The ability to inhibit mutation could be a possible mechanism that dairy derived LAB and milk proteins/peptides affect the development of cancer.

2.3.1 The Antimutagenic Properties of *Lactobacillus casei* ADA03 and Milk Proteins/Peptides

In the present study, we found that various treatments of *L. casei* ADA 03 had significant antimutagenic activity against 2AF induced mutation of *Salmonella typhimurium* TA98 (Table 2.1). Inhibitory activity ranged from 36.8% to 51.6% (Figure 2.1). Live cells, 24 hour fermented milk and 7 Day fermented milk samples demonstrated statistically significant inhibitory activity. Interestingly, the peptidoglycan fractions did not exert any inhibitory activity, indicating perhaps that the antimutagenic activity against 2AF induced mutation is not due to cell wall fractions.

Others have found antimutagenic activity attributed to peptidoglycans isolated from LAB (Rajendran and Ohta, 1998; Asahar et al., 1992). However, they suggested that antimutagenic activity of LAB is due to binding and inactivating mutagens, thus preventing their interaction with target tissues. Live cells, pure cell wall and peptidoglycan derived from LAB isolated from Miso were able to bind to many foodborne mutagens (Rajendran and Ohta, 1998) and to prevent mutagen associated mutation in *Salmonella typhimurium* TA1535/pSK1002 (Asahara et al., 1992). Peptidoglycan harvested from *Streptococcus lactis* and *Streptococcus cremoris* had a strong binding activity to heterocyclic amines (Zhang and Ohta, 1991).

This discrepancy between our results and that of other researchers could be due to the inherent differences in activity between and among strains and species of LAB or to the carcinogenic agent we used. The mutagens these researchers used were foodborne mutagens, whereas 2AF is a highly carcinogenic synthetic chemical that is not normally found in nature. In addition, this suggests that peptidoglycan isolated from *L.casei* ADA 03 may not exert antitumour activity through antimutation but through alternate mechanisms, since studies previously performed in this laboratory demonstrated tumour inhibition by this strain (Macleod et al., 1990).

In the present study, we found that *L. casei* ADA 03 fermented milk had significant antimutagenic activity. The 24 hour fermented milk had 51.6% antimutagenic activity, whereas antimutagenic activity decreases slightly to 45.4% after 7 days incubation. However, there was not a statistically significant difference between these two treatments. The slight decline in antimutagenic activity could be due to a change in the protein/peptide profile as a function of increasing fermentation time. In addition, after 7 days incubation, perhaps the viability of live cells has decreased slightly resulting in lessened antimutagenic activity. However, researchers have shown that yogurt/fermented drinks, when stored at 6 °C and 12 °C still have a 10^7 CFU/g of LAB after 60 and 45 days storage, respectively (Birollo et al., 2000). Regardless, we must allow for differences between strains of bacteria and the storage conditions for the present experiment as compared to that of Birollo et al.

Indeed, other researchers have found similar results. Extracts of milks fermented with *Streptococcus thermophilus, Lactobacillus bulgaricus* or a combination of both were tested for their ability to prevent mutation induced by mutagens, 4-nitroquinoline-*N*-oxide (NQNO) and 2AF. They found that antimutagenic activity was the highest in milks fermented with both strains against both mutagens, whereas unfermented milk extracts had no activity (Bodana and Rao, 1990). Cassand et al. (1994) observed antimutagenic activity of milks fermented with *Bifidobacterium* and *Lactobacillus* spp. in *Salmonella typhimurium* TA98 and TA100. These strains had significant antimutagenic activity against direct acting chemical mutagens NQNO and 2-nitrofluorene (NF) and against indirect acting mutagens of dietary origin, a polycyclic hydrocarbon formed during flavonoid present in vegetables (quercetin). Cell free supernatants collected from LAB isolated in Italian dairy products (*L.casei, L. acidophilus, L. delbrueckii* subsp. *bulgaricus, L. rhamnosus* and *L. plantarum*) had significant antimutagenic activity against mutations induced by 4-nitroquinoline-1-oxide (4NQO) and MNNG in *Salmonella typhimurium* TA 100 (Caldini et al., 2005).

It also has been established that LAB used to ferment food other than milk also possess antimutagenic activity. Kimchi, is a traditional vegetable pickle fermented by LAB that has been widely consumed in Korea for thousands of years. MRS broth supernatant of *L. plantarum* KLAB21 isolated from kimchi had significant antimutagenic activity against mutation induced by MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), NQO, NPD (4-nitro-*O*-phenylenediamine) and aflatoxin B1 in *Salmonella typhimurium* strains TA 100 and TA98. Furthermore, cell wall fractions from *L. plantaram* KLAB21 had significant, although lower, antimutagenic activity. Due to the low yield of the cell wall fractions, these researchers concluded that the majority of the antimutagenic activity is from an extracellular compound that is secreted into the culture medium (Park and Rhee, 2001). Indeed, three glycoproteins were isolated and purified from culture supernatant of *L. plantarum* KLAB21, had significant antimutagenic activity against MNNG induced mutation of *Salmonella typhimurium* TA 100 (Rhee and Park, 2001).

Dadih and terasi are traditional Indonesian fermented food products. Terasi is highly mutagenic to bacteria in the AMES test (Surono and Hosono, 1996). Dadih, on the other hand, is a fermented food product produced from 36 strains of LAB. LAB used in Dadih fermentation were inoculated individually into milk and tested for antimutagenic activity against terasi induced mutation in streptomycin-dependent strain 510 derived from *Salmonella typhimurium* TA 98. They found that all fermented milks displayed antimutagenic activity against terasi induced mutation, however, they found that a decrease in the number of revertants was only observed with pretreatment. This suggests that LAB act in a desmutagenic manner, by binding mutagenic terasi as opposed playing a role in DNA repair (Surono and Hosono, 1996).

We observed that the 24-hour and the 7-Day fermented milk samples had significant antimutagenic activity that was higher than the live cells. This indicates that in addition to live cells, fermentation of milk resulted in the liberation of bioactive peptides or fatty acids or the release of exogenous components from *L. casei* ADA 03. However, the 24 hour fermented milk supernatant had very low antimutagenic activity, indicating that the active components could be derived from the casein protein fraction.

It has been found by other researchers that through bacterial enzymatic digestion or lactic acid degradation of the native milk protein structure, bioactive proteins/peptides are liberated from their native structure (Law and Haandrikman, 1997; LeBlanc et al., 2002). In addition, lipases from LAB have been shown to release antimutagenic free fatty acids during milk fermentation. Fatty acids, in particular palmitic acid, have been reported as the major fatty acid responsible for antimutagenic activity against MNNG and 3,2'-dimethyl-4-aminobiphenyl (DMAB) induced mutation (Nadathur et al., 1998; Bakalinsky et al., 1996; Nadathur et al., 1996; Nadathur et al., 1995; Nadathur et al., 1994). Others have suggested that production of exopolysaccharides by bacteria and subsequent binding of carcinogens by exopolysaccharides contribute to antimutagenic activity (Sreekumar and Hosono, 1997; Rhee and Park, 2001).

To determine whether the antimutagenic component resides in the liberation of bioactive proteins and peptides from milk during fermentation, we tested several individual milk protein/peptide fractions (Table 2.1). Casein hydrolysate and sodium caseinate augmented mutagenic activity of 2-AF, whereas β-casomorphin-7 decreased the number of revertants. Although the antimutagenic activity of β-casomorphin-7 was not significant, mutation inhibition ranged from 23.7% to 32.6%. In addition, there was a negative correlation ($p \leq 0.05$) between β-casomorphin-7 concentration and mutation rate (Figure 2.2). Thus β-casomorphin-7's potential, as an antimutagenic agent should not be ignored.

Contrary to our results other researchers have found antimutagenic activity and binding of mutagens associated with casein (Tiedink et al., 1989; Berg et al., 1990; Goeptar et al., 1997; Yoshida and Xiuyun, 1992). Van Boekel et al. (1993), found that sodium caseinate and hydrolyzed casein had significant antimutagenic potential against benzo(a)pyrene, *N*-methylnitrosourea and nitrosated 4-chloroindole in the AMES reverse mutation assay. Perhaps, sodium caseinate and casein hydrolysate are not effective at blocking mutations induced by 2-AF, whereas with other mutagens it is effective. In addition, since casein hydrolysate is a mixture of different proteins/peptides, perhaps there are other peptides present that prevent casein from interacting with the mutagen; or perhaps the degree of hydrolysis was not high enough to liberate antimutagenic peptides from the native protein structure.

Indeed, van Boekel et al. (1993) observed that increasing hydrolysis of casein coincided with increasing antimutagenic activity. They suggested that hydrolysis increased the peptides formed and resulted in a better accessibility of casein peptides for interactions with mutagens. Indeed, our experiments demonstrate that β-casomorphin-7, an opioid peptide present in the native structure of β-casein, had significant antimutagenic activity against 2AF induced mutations whereas casein hydrolysate and sodium caseinate did not possess antimutagenic activity.

We also tested whey derived protein β-lactoglobulin. At higher concentrations (120 and 90 mg/mL), β-lactoglobulin was mutagenic towards *S.typhimurium* TA98, whereas at lower concentrations, although statistically insignificant, there was a reduction in the number of revertants (19.6% - 20.1%), i.e. mutation rate. Our results are similar to that of other researchers. Yoshida et al. (1991) demonstrated that β-lactoglobulin can dose dependently bind mutagenic heterocyclic amines, derived from protein cooked at high temperatures, Trp-P-1 (3-amino-1,4-dimethyl-*5H*-pyrido[4.3-*b*]indole), Trp-P-2 (3-amino-1-methyl-*5H*-pyrido[4,3-*b*]indole) and Glu-P-1 (2-amino-6-methyldipyrido[1,2-*a*:3'2'-*d*]imidazole). However, Yoshida found that after the optimum dose was achieved, antimutagenic activity did not decrease, as observed in the present experiment. Perhaps, β-lactoglobulin ability to prevent 2AF induced mutation is achieved by binding of the mutagen at lower concentration, however at higher concentrations of β lactoglobulin perhaps other pathways are activated resulting in further mutation.

In addition, we observed that live *L.casei* ADA 03 significantly decreased the number of histidine revertants induced by 2-AF. Live *L. casei* and Omniflora ®, a probiotic preparation often prescribed for stomach and intestinal disorders had significant activity against indirect acting mutagen beef extract and direct acting mutagen nitrosated beef extract, respectively (Renner and Munzner, 1990). Similarly, Ebringer et al. (1995) observed that *Enterococcus faecium* live cells but not dead cells decreased mutation induced by nitrovin and 2AF. They suggested that the antimutagenic activity of *E. faecium* is due to some factors within *E. faecium* to influence the metabolic transformation of mutagens. However from the current experiment, we can not determine how mutation was inhibited, just which components are able to induce antimutation.

Mutations occur when a DNA gene is damaged or changed through spontaneous mutation, chemical alteration or via viral induction, in such a way as to alter the genetic message carried by that gene. The uncontrolled proliferation of cells with mutated genes results in the formation of cancer. The present study and that of others demonstrates the potent ability of dairy derived LAB and proteins/peptides to inhibit chemically induced mutations in bacterial systems. The purpose of such studies in bacterial systems is to identify potential agents that could prevent DNA damage in more sophisticated systems. Substances with the ability to prevent DNA mutations are termed antigenotoxins. Perhaps it is through antigenotoxic action that LAB can exert an antimutagenic effect.

Indeed, recent studies demonstrate that LAB derived from various sources has antigenotoxic capabilities. *L. acidophilus* strain 317/402 commonly referred to as Narine is widely used for fermenting baby food in Armenia. Narine was able to prevent DNA damage in rodent gastric and colon cells induced by MNNG (Nersesyan, 2001). Furazolidone, broad-spectrum antibiotic used to treat infectious diarrhea and enteritis caused by bacteria or protozoans, can also cause mutations to DNA. Several strains of LAB (*Lactobacillus plantaram* KL and 8014, *Lactobacillus casei Shirota, Lactobacillus acidophilus* T_{20}, *Streptococcus salivarius* var. *thermophilus* and *Bifidobacterium lactis* Bb-12) all demonstrated significant antigenotoxic activity against furazolidine (Raipulis et al., 2005). LAB isolated from Italian dairy products had significant antigenotoxic activity against 4NQO and MNNG in Caco-2 enterocytes and in an *E.coli* strain. Moreover, strains found to have strong antigenotoxic activity also exhibited strong antimutagenic activity (Caldini et al., 2005; Cenci et al., 2002).

Research has demonstrated an epidemiological and *in vivo* link between cancer development and consumption of yogurt and yogurt related components, specifically LAB and bioactive milk proteins/peptides/fatty acids that are derived from milk during LAB fermentation. However, these studies, which involve complex systems with multiple variables, have not as of yet elucidated the precise mechanisms of action. Although bacterial assays are not as sophisticated as mammalian systems, they do provide us insight into possible mechanisms of action. The present study demonstrates that *L. casei* ADA 03 and several milk proteins may contribute to the anticancer effect by acting as antimutagenic compounds, perhaps through direct interaction with the mutagen or through prevention of DNA damage.

2.4 CONCLUSION

In the present experiment, we have demonstrated that *Lactobacillus casei* ADA 03 (live and fermented milk samples) has significant antimutagenic activity against 2 AF induced mutation of *Salmonella typhimurium* TA98. In addition, we have shown that bioactive proteins and peptides, such as β casomorphin-7 and β-lactoglobulin that are released from milk during fermentation or naturally present in milk also have antimutagenic activity. Mutations to DNA can ultimately result in the development of cancer. The ability to inhibit mutation or to augment the body's DNA repair system by consuming a dairy product with probiotics and bioactive proteins/peptides has a significant economic impact upon dairy industry and therapeutic and economic impact upon the health care system. Further research is required to fully characterize the health benefits of dairy derived LAB and protein/peptides; with the ultimate goal being the development of a dairy based product that can act as a dietary immunoadjuvant in cancer prevention/treatment.

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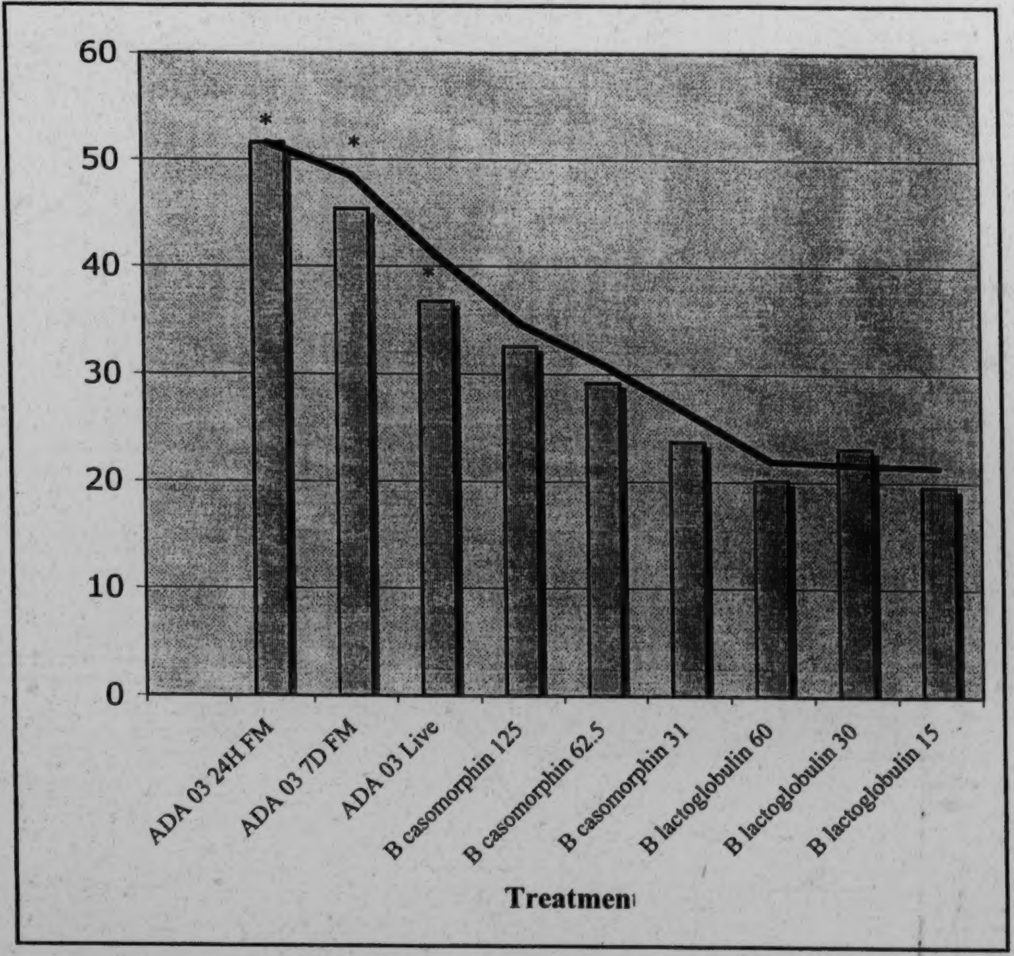
Table 2.1 Antimutagenic Activity of Dairy Derived LAB and Protein/Peptides against 2-Aminofluorene induced mutation of *Salmonella typhimurium* TA 98

LAB/Milk Protein/Peptide	Treatment/Concentration	*Salmonella typhimurium* TA 98 Count[a]	*Inhibition Augmentation*[b]
Control	**Positive**	323.6±72.1	0
	Negative	20.1±3.8	93.8
	Untreated	22.8±3.8	93.0
***Lactobacillus casei* ADA 03**	**Live**	204.6±23.7*	36.8
	24 Hour Fermented Milk	156.7±40.7*	51.6
	7 Day Fermented Milk	176.6±33.0*	45.4
	Peptidoglycan 1000 μg/mL	327.0±30.9	*1.1*
	Peptidoglycan 800 μg/mL	339.8±7.5	*5.0*
	24 hour Fermented Milk Supernatant	338.7±35.8	*4.7*
β-Casomorphin-7	**125 μg/ml**	218.1±14.8	32.6
	62.5 μg/mL	229.2±5.4	29.2
	31.25 μg/mL	246.9±14.4	23.7
β-Lactoglobulin	**120 mg/mL**	450.8±9.4	*39.3*
	90 mg/mL	569.6±40.8¥	*76.0*
	60 mg/mL	258.7±35.8	20.1
	30 mg/mL	248.9±45.5	23.1
	15 mg/mL	260.3±37.5	19.6
Casein Hydrolysate	**40 mg/mL**	603.0±116.9¥	*38.9*
	20 mg/mL	476.1±149.6¥	*47.1*
	10 mg/mL	449.4±126.0¥	*86.3*
Sodium Caseinate	**75 mg/mL**	374.7±26.2	*15.8*
	37.5 mg/mL	323.9±47.3	*0.1*
	18.75 mg/mL	345.2±11.5	*6.7*

* Significantly different from control ($p \leq 0.05$); ¥ Significantly higher than the control ($p \leq 0.05$)

a: Each experiment (minimum n=3) was performed in triplicate; b:Augmentation: values are italicized

Figure 2.1 Inhibition of Mutagenic Activity Induced by 2-Aminofluorene in *Salmonella typhimurium* TA 98[a]

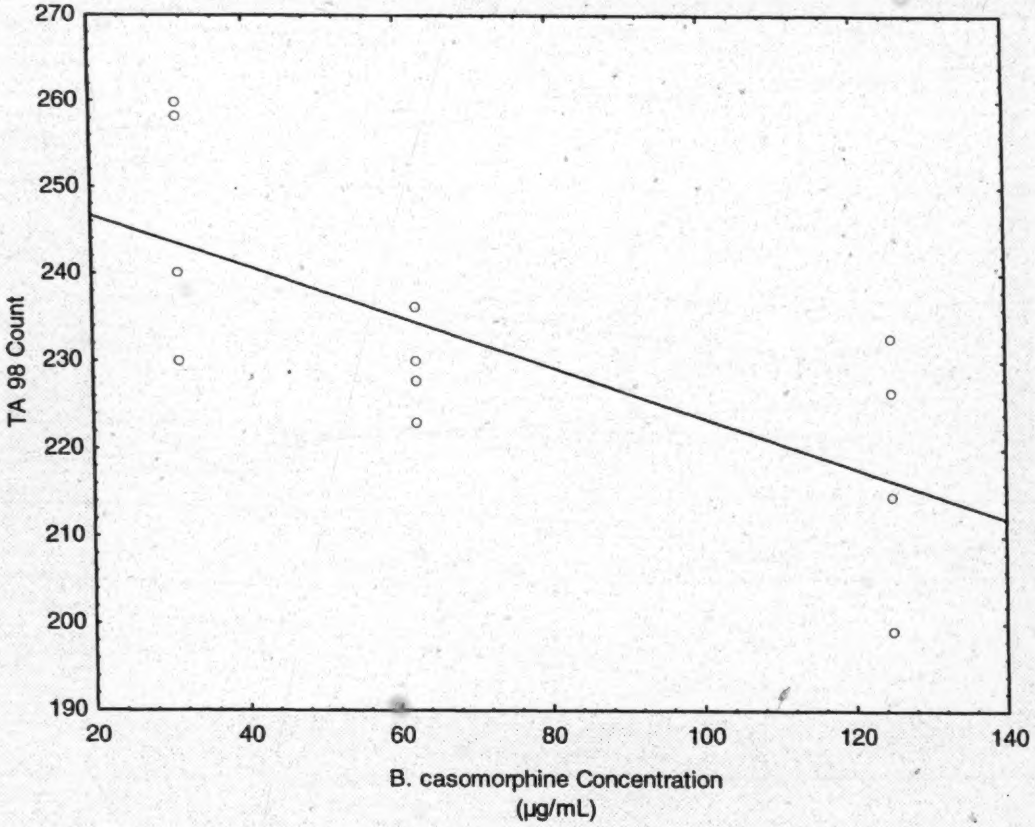


*Significantly different from control ($p \leq 0.05$)

Abbreviations: 24 H FM: 24 hour fermented milk; 7D FM: 7 day fermented milk

[a] Using the Ames reverse mutation assay, overnight cultures of *Salmonella typhimurium* TA 98 were incubated with the previously described treatments for 48-72 hours. The number of revertants was counted using an automatic colony counter.

Figure 2.2. Effect of β-casomorphin-7 concentration on the antimutagenic response.



CHAPTER 3[*]

Adhesion of Various Strains of Dairy Derived Lactic Acid Bacteria to Human Colon Cancer Cell Line HT29

3.1 INTRODUCTION

Although the popular use of probiotics has only gained momentum within the last two decades, the history of its usage is quite long. There are records dating back as far as 76 B.C. when the Roman historian Plinius recommended the administration of fermented milk products for the treatment of gastroenteritis (Schrezenmeir and de Vrese, 2001). Nonetheless, Metchnikoff (1907) is often credited for popularizing the concept that using probiotics or lactobacilli results in the reduction of toxin producing-bacteria in the gut and that this increases the longevity of the host. However, the term probiotic, Greek meaning for life was first used by Lilly and Stillwell (1965). They described probiotics as "substances secreted by one microorganism which stimulates the growth of another". This definition was revised by Fuller to (1989) "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." However, Salminen (1999) has further refined this definition to a microbial cell preparation or components of microbial cells that have a beneficial effect on the health and well being of the host.

Probiotics have been reported to have beneficial effects upon the host in many diseases and disorders. Probiotics have been shown to exert antimicrobial effects upon pathogenic bacteria (de Vrese and Schrezenmeir, 2002); in fact gastrointestinal bacteria are the first line of defense against assault by pathogenic bacteria (Salminen et al., 1996). In addition probiotics aid in lactose digestion (de Vrese et al., 2001), reduction in cholesterol and blood pressure (Lin et al., 1989; Hata et al., 1996), management of diarrhea (Raza et al., 1995) and antitumour properties (Aso et al., 1995; Macleod, 1990). The current consensus is that probiotic bacteria are to be viable in order to exert any beneficial effect, although there have been many studies that demonstrate the health promoting effects of nonviable or cell wall components of lactic acid bacteria (LAB) (Korhonen et al., 2001; Wallace et al., 2003; Haller et al., 1999).

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Regardless, adhesion aids the bacteria in transient colonization of the gut preventing rapid removal by peristaltic contractions. In addition, adhesion would increase the residency time of LAB within the gut, to allow maximum time for probiotic effects. Thus adhesion to the gut is one of the primary selection criteria for a strain of LAB to be classified as a probiotic (Tuomola et al., 2001). The measurement of adhesion *in vivo* is confounded by many factors, thus reliable *in vitro* adherence assays have been developed (Blum et al., 1999; Sarem-Damerdji et al., 1995).

This current study was undertaken to investigate the adhesive properties of dairy derived LAB (*Lactobacillus acidophilus* MR 100, *Lactobacillus bulgaricus* MR 110, *Lactobacillus acidophilus* NRC 13017, *Lactobacillus acidophilus* NRC 13019, *Lactobacillus casei* ADA 03, *Lactobacillus casei* NRC 13005, *Lactobacillus casei* subsp. *casei* CSCC 2601 and yogurt starter culture (YC 085)) to human colon cancer cell line HT29.

3.2 MATERIALS AND METHODS

3.2.1 Probiotic LAB Propagation and Growth

Experimental strains used for adhesion testing were obtained from stock cultures available in our laboratory. They were cultured in MRS broth (Difco, Detroit, MI, USA) supplemented with 20% (v/v) sterile reagent grade glycerol (Sigma Aldrich, St. Louis, MO) as a cryoprotective agent, and stored at –80 °C until required for use in experiments.

A 1% (v/v) inoculum of each LAB strain from frozen stock cultures were grown in lactobacilli MRS broth (37 °C for 24 hours) and used immediately for testing. Experimental cultures were subcultured twice to minimize the amount of cryoprotective agent in the final incubation mixture.

3.2.2 HT29 Human Colon Cancer Cell Line Growth and Propagation

HT29 human colon cancer cell line (ATCC HTB-38) was kindly provided by Joan Turchinsky (Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, Alberta). HT29 cells were grown following the procedure recommended by ATCC. Briefly, an aliquot of HT29 frozen stock cultures (-80°C) was grown in 75 cm^2 vented tissue culture flasks (Corning, Fisher Scientific, Nepean, ON, Canada) with McCoys 5A Modified Medium (with 1.5 mM L-glutamine/0.026 M sodium bicarbonate; Sigma Aldrich) supplemented with 10% fetal calf serum (FCS; Gibco Laboratories, Chagrin Falls, IL, USA).

HT29 cells were routinely grown at 37°C with 5% CO_2/95% air atmosphere incubators. Spent tissue culture media was replaced every 2 to 4 days, depending upon cell culture density. Cells were routinely passaged with 0.25% trypsin – 0.53 mM EDTA solution (Sigma Aldrich) when monolayers reached 80% confluence. Cell number and viability were assessed using trypan blue exclusion with a haemocytometer. Aliquots (1mL) of HT29 stock cultures suspended in FCS (Gibco) supplemented with 10% tissue culture grade DMSO (Sigma Aldrich) were stored at -80°C until required for experimental use or for routine subculturing.

3.2.3 Adhesive Ability of Probiotic LAB to HT29 Human Colon Cancer Cell Line

3.2.3.1 Biochemical Characterization of Adherence

We tested the effect of various chemical and physical treatments on adhesive properties of dairy derived LAB. Trypsin, sodium metaperiodate, ethylenediamenetetraacetic acid (EDTA) and [ethylene-bis-(oxyethylenenitrilo)] tetraacetic acid (EGTA) were all purchased from Sigma Aldrich.

To determine whether adhesion was due to a proteinaceous ligand, overnight bacterial cultures (1mL) were washed three times with PBS to remove spent culture media. The bacterial cultures were resuspended in 1mL MRS broth with trypsin (2.5 mg/mL) for 1 hour (37°C). The trypsin was inactivated by the addition of 1 mL of FCS prior to adherence assays.

To determine if adhesion was due to a carbohydrate associated cell surface component, bacterial cultures were preincubated for 30 minutes with 1 M metaperiodate solution. The cells were then washed three times with PBS and then resuspended in tissue culture media and used for adhesion assays.

To determine whether adhesion was due to divalent cations, which are cofactors involved in enzymatic reaction, the HT29 monolayers were washed 5 times with 20 mM EDTA or 20 mM EGTA in PBS after incubation with bacterial cultures (Coconnier et al., 1992).

3.2.3.2 *In vitro* Adhesion Assay: Light Microscopy

Adhesion testing was done as per methods described by Gopal et al., (2001) with slight modifications. Briefly, HT29 cells were seeded onto sterile glass coverslips in 6 well plates at a concentration of 6.3×10^4 cells per well. These cells were incubated (37°C in 5% CO_2/95% air) overnight to allow for attachment. The HT29 cells were washed with sterile PBS three times to remove spent cell culture media. Two mL of overnight bacterial cultures were transferred into sterile centrifuge tubes (Fisher Brand, Nepean Ontario, Canada), then centrifuged at 10 000 g for 1 minute. The bacterial cells were then washed with sterile PBS. This was repeated three times. The bacterial cell pellet was then resuspended into 2 mL McCoys modified 5A media (supplemented with 10% FCS) and used immediately.

One mL of the untreated or chemically/physically treated bacterial cultures were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). After 1 hour of incubation, the monolayers were washed three times with sterile PBS to remove non-adherent bacteria. The monolayers were then fixed with methanol (3 mL/well) for 5 minutes, and Gram stained according to the manufacturer's (Sigma Aldrich) protocol. The Gram stained cells, were examined by light microscopically. Each experiment was performed three times in triplicate and for each sample, 20 microscopic fields were counted.

3.2.3.3 Adhesion Testing: Scanning Electron Microscopy

HT29 cells grown on sterile glass coverslips were used for scanning electron microscopy (SEM). As previously described, bacterial cells were incubated with HT29 cells in 6 well plates (6.3×10^4 cells per well), and allowed to incubate for 1 hour (5% CO_2/37 °C). After the 1 hour incubation period, the cell culture was washed four times with PBS to remove all residual media and non-adhering bacteria. The cells in each well were then fixed with 2.5% (v/v) glutaraldehyde (Sigma Aldrich) in 0.1 M phosphate buffer, pH 7.4, at room temperature for 1 hour. The cells were then washed with 0.1 M phosphate buffer, pH 7.4, two times to remove residual glutaraldehyde. After washing, the cells were post fixed for 30 minutes at 37°C with 2% (w/v) osmium tetraoxide (Sigma Aldrich; in 0.1 M phosphate buffer, pH 7.4). The cells were then washed three times with the same buffer. The cells were dehydrated in a graded series of ethanol (30, 50, 70, 80, 90, 98% v/v) and then passaged through a graded series of amyl acetate (50, 70, 90, 100% v/v in ethanol). The cells were then dried in a critical point dryer (Polaron Jumbo) and stored in a vacuum dessicator until used for SEM. Prior to SEM the cells were coated with gold (SEMprep 2, Nanotech) and the specimens were examined via SEM (JEOL JSM 6301FXV) to determine adhesion (Kociubinski et al., (2002).

3.2.4 Acid and Bile Tolerance Testing of Potential Probiotic LAB

Crude tests were performed to determine if each organism was acid tolerant. Overnight cultures of each LAB (1 mL) was centrifuged at 10 000 g for 1 minute. The cells were then washed three times with sterile PBS to ensure complete removal of cryoprotective agent from the incubation mixture. The pellet of bacterial cells obtained after centrifugation was then resuspended in PBS (1mL). An aliquot (100 µL) of the bacterial suspension was inoculated into 5 mL of acidified MRS broth (AMRS) in a tube. Four preparations of AMRS each adjusted to pH 6.8, 3.5, 2.5 and 1.5, respectively, were used in this experiment. The tubes were then incubated at 37°C for 24 hours, and visually examined for growth; for pH 1.5 the cell suspension was measured spectophotometrically since there was no obvious visible growth. The tubes without growth were incubated for another 24 hours to check growth of cells.

To emulate physiological gastrointestinal conditions, sequential acid and bile tolerance testing was performed. Overnight cultures of LAB (1mL) was centrifuged at 10 000 g for 1 minute and washed with sterile PBS. This was repeated 3 times. The pellet of LAB obtained after centrifugation was then resuspended into 1 mL of acidified AMRS broth (pH 2.5). The culture was then incubated at 37°C for 1 hour using a shaking water bath. After incubation, the cells were centrifuged (10 000 g for 1 minutes) and washed with sterile PBS. This was repeated 3 times. The pellet was then resuspended into 1 mL of Oxgall bile (Difco) and incubated at 37°C for 1 hour using a shaking water bath. After bile treatment, the cells were centrifuged (10 000 g; 1 minute) and washed with sterile PBS. This procedure was repeated 3 times. The pellet of cells obtained was then resuspended into 1 ml of standard MRS broth (pH 6.5); 100 µL of this bacterial suspension was inoculated into 10 mL of standard MRS broth. The cells were incubated at 37°C for 24 hours. After incubation, the tubes were visually inspected for growth of cells. If bacterial growth was not visually detected, growth was measured using a spectrophotometer (650 nm).

3.2.5 Statistical Analysis

Statistical Analysis was carried out in the Statistica program (Statistica 6.0). The data was analyzed through analysis of variance, Tukey's test, correlation of analysis and regression analysis.

3.3 RESULTS AND DISCUSSION

It has long been considered as a requirement that probiotic bacteria adhere to the target tissue within the gastrointestinal system in order to exert an effect. Therefore it is important to test potential strains for particular characteristics. Using *in vitro* testing, eight potential strains of *Lactobacillus* were examined for their ability to adhere to enterocyte-like HT29 human colonic cells. In addition, chemical treatments were administered to determine the mechanisms of adhesion.

3.3.1 Acid and Bile Tolerance of Dairy Derived LAB

We simply tested the acid and bile tolerant properties of each of the LAB used in this experiment. We found that each strain grew well at pH 6.8 to 2.5, whereas at lower pH 1.5 there was no growth (Table 3.1). The results are in good agreement with other researchers (Jacobsen et al., 1999; Baccigalupi et al., 2005). Mishra and Prasad (2005) found that seven strains of *L. casei* could not survive growth at pH 1 and only three strains survived at pH 2. The results in the present experiment demonstrate that growth is not affected at $pH \geq 2.5$. This is important because Martini et al., (1987) demonstrated that during the ingestion of yogurt or milk gastric decreases from $pH \geq 5$ to around 3, stressing the importance of bacteria being able to survive at $pH \geq 2.5$.

In addition, we tested the ability of the bacteria to survive sequential treatments of acid and then bile, to mimic gastrointestinal conditions. Based on the visual examination, we found that each strain was hardy and survived treatment with acid and bile (Table 3.2). This indicates that these strains would be able to survive the harsh conditions of the gastrointestinal tract to reach the final destination of action, the colon. Since acid and bile tolerance testing was not our primary objective, we did not use more sophisticated methods to determine tolerance. In addition, many researchers have reported that viability is not a necessary requirement for probiotic actions (Korhonen et al., 2001; Wallace et al., 2003; Haller et al., 1999). Indeed, experiments performed in this laboratory demonstrate that heat killed cells and peptidoglycan have profound effects upon tumour necrosis factor, interleukin 6 and nitric oxide secretion from macrophages.

3.3.2 Adhesion of Dairy Derived LAB to HT29 Colon Cancer Cells

In the present study, we measured adhesion by two methods: Gram staining and SEM. Through simple Gram staining, we observed that all the strains tested had strain dependent adhesion properties to HT29 colon cancer cells (Table 3.3). Adhesion criteria were those set out by Jacobsen et al. (1999). As shown in Table 3.3 some strains were able to adhere to HT 29 cells more efficiently than the other strains. The degree of adhesion was the highest ($p \leq 0.05$) in CSCC 2601, and higher ($p \leq 0.05$) in YC 085 than in ADA 03 and ADA 05. There was, however, no difference ($p \geq 0.05$) in adhesiveness between the latter two strains. The results also demonstrated that the degree of adhesiveness was similar among ADA 05, NRC 13109, and MR 110 but higher ($p \leq 0.05$) in ADA 05 compared to MR 100 and NRC 13017 with no difference ($p \geq 0.05$) being observed among the four strains including MR 100, MR 110, NRC 13017 and NRC 13019.

These results are in good agreement with other researchers. Morita et al. (2002) examined the attachment of 30 strains of LAB from dairy and human intestinal origin. They found that most strains had adhesive properties, however, they observed that the majority of strains of dairy origin only had slight adhesion to Caco2 cells (1-20 bacterial cells/microscopic field). The adhesion qualities of 47 strains of LAB from human, dairy and other food origins was tested by Jacobsen et al. (1999). The majority of the strains tested had low to moderate adhesion, however a few strains tested had comparable results to our experimental strains. The highest adhesion observed by these researchers was found in *L. rhamnosus* GG (LGG) and *L. rhamnosus* 19070-2 (19070-2) both of human origin. However, dairy strain *L. casei* subsp. *alactis* CHCC 3137 had similar adhesion counts of 551± 223 compared to LGG (630±275) and 19070-2 (713±188) (Jacobsen et al., 1999).

The strains that we tested had much greater adhesive qualities than the dairy LAB studied by Morita et al. (2002) and by Jacobsen et al., (1999). Although all the strains, with the exception of *L. acidophilus* NRC 13017, all demonstrated strongly adhesive qualities, CSCC 2601, YC 085, ADA 03 and NRC 13005 showed the highest affinities for binding to HT 29 cells. Moreover, as compared to LGG counts (713±188) from Jacobsen et al., (1999) *L. casei* ADA 03 had similar counts (780±283.3), whereas YC085 and CSCC 2601 had dramatically higher adhesion counts. This is significant because *L. rhamnosus* GG is very well studied with documented properties (Korhonen et al., 2001; Schultz et al., 2004).

Furthermore, as demonstrated by Morata de Ambrosini (1998, 1999) adhesion is a key determinant to immunostimulatory activity, thus it would be logical to assume that CSCC 2601, YC 085 and ADA 03 would have significant immunostimulatory activity. Indeed, ADA 03 was found to have significant killing activity towards Morris Hepatoma Cancer cells *in vivo* (Macleod et al., 1990) and further studies performed in our laboratory demonstrate that these strains have immunostimulatory activity, with respect to nitric oxide, tumour necrosis factor and interleukin 6 (unpublished data). However, Morita et al., (2002) did not investigate a similar connection between the degree of adhesion and immunostimulatory activity.

The results from the present experiment may differ from those of Morita et al., (2002) and Jacobsen et al., (1999) due to the inherent differences between the strains we tested; in addition, since we used HT 29 human colon cancer cell line instead of Caco-2 cells, perhaps the dairy LAB we tested had greater affinity for HT 29. Caco-2 and HT 29 cells are both colon adenocarcinoma cells of human origin. However there are differences between the cells. Caco-2 cells are from a 72 year old male subjects, whereas HT 29 was isolated from a 44 year old female. Indeed, Conconnier et al. (1992) demonstrated different adhesion patterns of LAB to Caco-2 than to HT 29 colon cancer cells.

Indeed, Mishra and Prasad (2005) observed a wide variation in adhesive qualities of seven strains of *Lactobacillus casei* of dairy origin. Bouzaine et al. (2005) studied the adhesion qualities of ten strains of LAB (five *Lactobacillus* spp., two *Weissella* spp. and three *Enterococcus* spp.) isolated from the gastrointestinal flora of broiler chickens. These authors reported that the majority of the strains tested were very weakly or no adherent. However, they found that two strains, including *Lactobacillus rhamnosus* TB1 and *L. reuteri* LRT1 were highly adherent. *L. rhamnosus* TB1 demonstrated extremely high attachment of cells (2×10^3 cells per microscopic field). In their vivo experiments, Bouzaine et al. (2005) confirmed attachment of both LRT1 and TB1 to intestinal epithelial cells with a strong affinity for ileum, jejunum and rectal tissues.

When Gram stained samples were examined by light microscopy, adherence occurred in chains and bacterial clusters, indicating bacterial cell to cell interactions occurring in addition to interactions with the intestinal epithelial cell surface (Figure 3.1 to 3.3). Unfortunately, due to the adherence patterns of bacteria to HT29 cells in clusters, counting was quite difficult and could not be done reliably (Figure 3.1). For example, counts of *L. casei* ADA 03 ranged from 130 to >1000. Regardless, differences between bacteria that were highly adhesive and those that were not as adhesive were quite obvious. These observations are in good agreement with Tuomola and Salminen (1998) and Conconnier et al. (1992). However, to confirm adhesion, HT29 cells incubated with LAB were examined with SEM.

SEM (Figures 3.4 – 3.10) confirms the attachment of dairy derived LAB to HT29 cells. Figures 3.4, 3.5 and 3.8 demonstrate the close association between *L.casei* subsp. *casei* CSCC 2601 and the microvilli of human colon cancer cell line HT29. Others have confirmed attachment of dairy derived propionibacteria to epithelial cells lines in the same manner (Huang and Adams, 2003).

However, Lee et al. (2000) demonstrated that there are only a finite number of bacterial receptors on intestinal surfaces for bacterial attachment, indicating the possibility of competitive adhesion. Indeed, Lee et al. (2000) observed that *L.rhamnosus* GG was not able to competitively inhibit *E.coli* attachment to intestinal epithelial cells. They suggest that *L. rhamnosus GG* has a high dissociation constant, thus any dissociated *L. rhamnosus* are quickly and readily replaced by pathogenic *E.coli.* However, with *L. casei* strain Shirota, the number of bound *E. coli* was significantly lower than with *L. rhamnosus* GG. They suggest that *L. casei* strain Shirota, has a lower dissociation constant and also produces a soluble protein factor that may hinder their displacement by *E. coli* cells (Lee et al., 2000). Moreover, *L.rhamnosus* GG was not able to affect the adhesion or viability of *S. enterica* serovar *typhimurium*; in fact the pathogen was shown to decrease *L. rhamnosus* GG adhesion (Versterlund et al., 2005). Thus it can be concluded that the concentration of free non-adhering *Lactobacillus* in the gastrointestinal contents needs to be maintained at a high level to prevent the adhered lactobacilli from being replaced by other bacteria.

3.3.3 Mechanisms of Adhesion: Chemical Treatments

We also studied the elements that are involved in the adhesion of these particular dairy derived strains from LAB. The gastrointestinal tract is a complex system, where cell to gastrointestinal surface interactions are speculated to be mediated by interactions occurring between specific molecules on bacterial cells and the gastrointestinal surface of the host. It has been suggested that lactobacilli adhere through their proteinaceous (Henricksson et al., 1991) or carbohydrate (Morata de Ambrosini et al., 1999) associated bacteria cell surface ligands. The difference in adhesion surface ligands between bacterial strains could explain species specificity in adherence to intestinal epithelial cells. Thus each strain of bacteria was treated with trypsin, sodium metaperiodate, EDTA and EGTA (see Methods) to further elucidate if attachment is through proteinaceous or carbohydrate based adhesion or if calcium and other divalent cations are involved in attachment (Table 3.1).

EDTA and EGTA are chelators of divalent cations and calcium, respectively (Table 3.1). Several strains tested demonstrated significant decreases in adherence after washing HT29 colon cancer cells with EDTA. Decreases in adhesion ranged from 58% in NRC 13017 to 95% in NRC 13019. With respect to EGTA, decreases in adhesion ranged from 97% in MR 100 to nearly 100% reduction in YC 085. The reduction in adherence after the administration of EDTA and EGTA indicate that divalent cations are also involved in bacterial cell to intestinal epithelial surface interactions. Conconnier et al. (1992) observed similar adhesion characteristics for *L. acidophilus* BG2FO4 to HT29 and Caco 2 cells.

Furthermore, pretreatment of LAB strains with proteinase, trypsin, significantly ($p \leq 0.05$) decreased the number of adhering bacteria in MR 100, ADA 03, CSCC 2601, NRC 13005 and YC 085. After pretreatment of the bacterial cells with trypsin, adhesion was less 88% in ADA 03 to 95% as observed with YC 085. This is indicative of a proteinaceous compound involvement in adhesion.

Pretreatment of LAB strains with sodium metaperiodate, significantly ($p \leq 0.05$) decreased adhesion counts of MR 100, NRC 13019, ADA 03, CSCC 2601 and YC 085. Decreases in adhesion ranged from 86% in *L. casei* ADA 03 to 97% reduction in YC 085; suggesting the involvement of carbohydrate associated compounds in adhesion of these strains to intestinal epithelial cells. Furthermore, several of these strains demonstrated significant protein/carbohydrate dependent adhesion. MR 100, ADA 03, CSCC 2601 and YC 085 all demonstrated significant decreases in adhesion numbers after the addition of trypsin and sodium metaperiodate. This suggest that a key factor involved in the adhesion of these strains to intestinal cell surfaces is a glycoprotein.

Interestingly, pretreatment of *L. casei* NRC 13005 with sodium metaperiodate augmented adhesion as compared to the control but not significant ($p \geq 0.05$). NRC 13005 adhesion was nearly doubled after treatment with sodium metaperiodate. But this was not statistically significant ($p \geq 0.05$). Perhaps some carbohydrate related substance provided competition for binding sites or had provided some form of steric hindrance resulting in further adhesion once these factors were removed. Similar results were observed by Gusils et al. (1999) for chicken LAB isolates *L. fermentum and L. animalis* and by Morata de Ambrosini et al. (1999) in human isolate *L. casei* CRL 431. They treated *L. casei* CRL 431 cells with sodium metaperiodate, an oxidizing agent of cell surface carbohydrate moieties. These cells showed nearly 4 times greater adhesion than the control after treatment with the carbohydrate oxidizing agent. Indeed, they suggest that the oxidation process caused by sodium metaperiodate results in physical and chemical changes in the bacterial surface, which improve cell stability in suspension and could permit adhesion.

Similar to our studies, Lee (2005) demonstrated that LAB *Weissella kimchii* PL9023 adhered to vaginal cells. The treatment of bacteria with periodate and proteinase K or denaturation with heat significantly decreased *Weissella kimchii* binding to vaginal cells. This suggests that binding of *Weissella kimchii* is mediated by a cell adhesion factor that is a glycoprotein. Baccigalupi et al. (2005) determined that *L. fermentum* strain BIO-DRL36 adhesion was mediated by low molecular weight factors, both smaller than 3kDa that were loosely associated to the cell wall to be responsible for adhesion. Others have identified lipoteichoic acid embedded in the cell wall to be involved in the adhesion of *Lactobacillus johnsonii* La1 to Caco-2 cells (Granato et al., 1999) or the presence of lectin-like structures and the involvement of divalent cations in adhesion (Gusils et al., 1999). Indeed, lectin like structures has been found to emerge from the cell surface of *L. casei* CRL 431. Lectin-like structures have the ability to bind to hydrocarbons present on the surface of epithelial cells (Morata de Ambrosini et al., 1999). In addition, these researchers demonstrate that adhesion is also dependent upon pH, increasing pH results in a decreased level of adhesion (Lee, 2005; Baccigalupi et al., 2005; Granato et al., 1999).

Furthermore, Granato et al. (2004) identified elongation factor Tu, typically considered to be involved in protein translation and normally not found on the cell wall but in the cytoplasm, to be a mechanism of adhesion for *L. johnsonii* La1. *L. acidophilus* BG2FO4 bind to Caco-2 cells primarily through a proteinaceous compound that is secreted into the spent culture supernatant and secondarily via cell surface associated glycoproteins (Conconnier, 1992; Greene and Klaenhammer, 1994). Others have found cell surface associated adhesion factors that are not proteinaceous in nature as well as extracellular adhesion factors that are heat resistant and proteinaceous are involved in adhesion (Chauviere et al., 1992). Furthermore, others have demonstrated that the binding of LAB are carbohydrate dependent, where carbohydrate moieties composed of galactose, glucose, fructose, mannose, fucose and many other sugar specific adherence factors are key elements in attachment of LAB to intestinal cell walls (Adlerberth et al., 1996; Lee and Puong, 2002).

3.4. CONCLUSION

Thus it can be seen from this present experiment LAB interact and bind with intestinal cells through a variety of mechanisms. Although there has not been a complete consensus reached on the requirement of adherence to initiate physiological effects, it appears that adherence to the target site of action allows for increased transient colonization of the site. In effect, this allows for increased reaction time. In the current experiment we have found that dairy derived LAB are able to adhere to HT 29 colon cancer cell line in a strain dependent manner, as confirmed by light and scanning electron microscopy. Furthermore, we have identified several adherence factors associated with each strain, however, that adherence mechanisms are very much strain dependent.

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Table 3.1: Tolerance of Lactic Acid Bacteria to Various Acidic Conditions

	Growth/Survival			
Strain↓ Condition→	**pH 6.8[a]**	**pH 3.5**	**pH 2.5**	**pH 1.5**
Lactobacillus acidophilus MR100	+	+	+	-
Lactobacillus bulgaricus MR110	+	+	+	-
Lactobacillus acidophilus NRC 13017	+	+	+	-
Lactobacillus acidophilus NRC 13019	+	+	+	-
Lactobacillus casei ADA 03	+	+	+	-
Lactobacillus casei ADA 05	+	+	+	-
Lactobacillus casei subsp. casei CSCC 2601	+	+	+	-

+ Indicates that there was growth

- Indicates that there was not any growth

[a] Lactic Acid Bacteria were incubated in acidified or standard (pH 6.8) MRS broth for 24 hours and measured for growth visually or with a spectrophotometer (650 nm).

Table 3.2: Tolerance to Sequential Acid and Bile[b] Treatments in Various Strains of Lactic Acid Bacteria[c]

Strain	Growth/Survival[a]
Lactobacillus acidophilus MR100	+
Lactobacillus bulgaricus MR110	+
Lactobacillus acidophilus NRC 13017	+
Lactobacillus acidophilus NRC 13019	+
Lactobacillus casei ADA 03	+
Lactobacillus casei ADA 05	+
Lactobacillus casei subsp. casei CSCC 2601	+

+ Indicates that there was growth

a: pH used for testing was pH 2.5. This was determined to be the lowest pH at which the bacteria were able to survive treatment, as determined by the Preliminary Acid Tolerance Testing

b: Oxgall Bile: Prepared according to manufacturers insert.

c. Lactic acid bacteria were incubated in 1 mL of acidified MRS broth at 37°C for 1 hour using a shaking waterbath. The cells were then washed with PBS and resuspended in Oxgall bile (1 mL). The bacteria were incubated at 37°C for 1 hour. The pellet was then resuspended into standard MRS broth and grown overnight. Growth was inspected visually and measured with a spectrophotometer (650 nm)

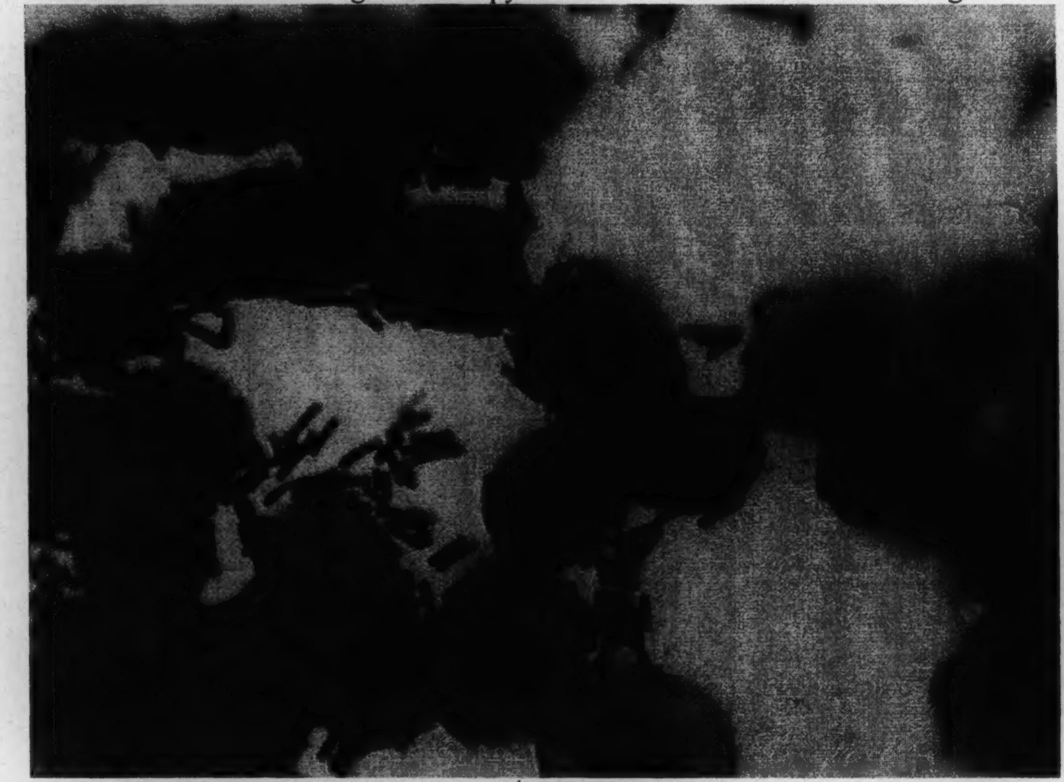
Table 3.3: Adhesion Properties of Several Potential Probiotics

Lactobacillus strain	NO TREATMENT*		CHEMICAL OR PHYSICAL TREATMENT$^{\Psi}$					
	Adhesion		Adhesion (Chemical or Physical Treatments)					
	Number of Adhering Bacteria*	Adhesiveness**	Control	EDTA	EGTA	Sodium metaperiodate	Trypsin	Adhesion Factor
Lactobacillus acidophilus MR100	155±63.9^{a}	++	155±63.9^{a}	210±68.1^{a}	2±0.7^{b}	17±6.87^{b}	14±1.7^{b}	Glycoprotein, Calcium
Lactobacillus bulgaricus MR110	259±166.3^{ab}	++	259±166.3^{a}	90±6.8^{a}	17±4.3^{a}	50±14.4^{a}	158±3.65^{a}	N/A
Lactobacillus acidophilus NRC 13017	91±39.4^{a}	+	91±39.4^{a}	38±5.9^{ab}	3±0.7^{b}	100±11.6^{a}	11±0.3^{ab}	Divalent Cation
Lactobacillus acidophilus NRC 13019	340±65.7^{ab}	++	340±65.7^{a}	15±2.1^{b}	5±1.3^{b}	16±9.0^{b}	359±165.5^{a}	Divalent Cation, Carbohydrate
Lactobacillus casei ADA 03	780±283.3^{c}	++	780±283.4^{a}	115±18.5^{b}	18±4.4^{b}	107±7.5^{b}	56±2.9^{b}	Glycoprotein, Divalent Cation
Lactobacillus casei NRC 13005	595±370.7^{bc}	++	595±370.7^{ab}	102±20.1^{b}	14.0±1.0^{b}	1156±779.7^{a}	74±39.1^{b}	Divalent Cation, Protein
Lactobacillus casei subsp. casei CSCC 2601	1986±926.1^{e}	++	1986±926^{a}	N/D	N/D	255±25.9^{b}	178±6.3^{b}	Glycoprotein
Yogurt Culture YC085	1400±266.8^{d}	++	1400±266.8^{a}	174±9.2^{b}	8±2.9^{b}	42±33.9^{b}	72±2.2^{b}	Glycoprotein, Divalent Cation

* No Treatment: Average number of adhering lactobacilli in 20 microscopic fields ± standard deviation (minimum n=13); Means in the same column with different letters are significantly ($p \leq 0.05$) different ($p \leq 0.05$). ** Adhesiveness criteria (modified from Jacobsen et al., 1999): No adhesion (- -): 0 bacteria; Non-adhesive: (-) 1-40 bacteria; Adhesive: (+) 41-100 bacteria; Strongly Adhesive: (++) >100 bacteria present in 20 microscopic fields

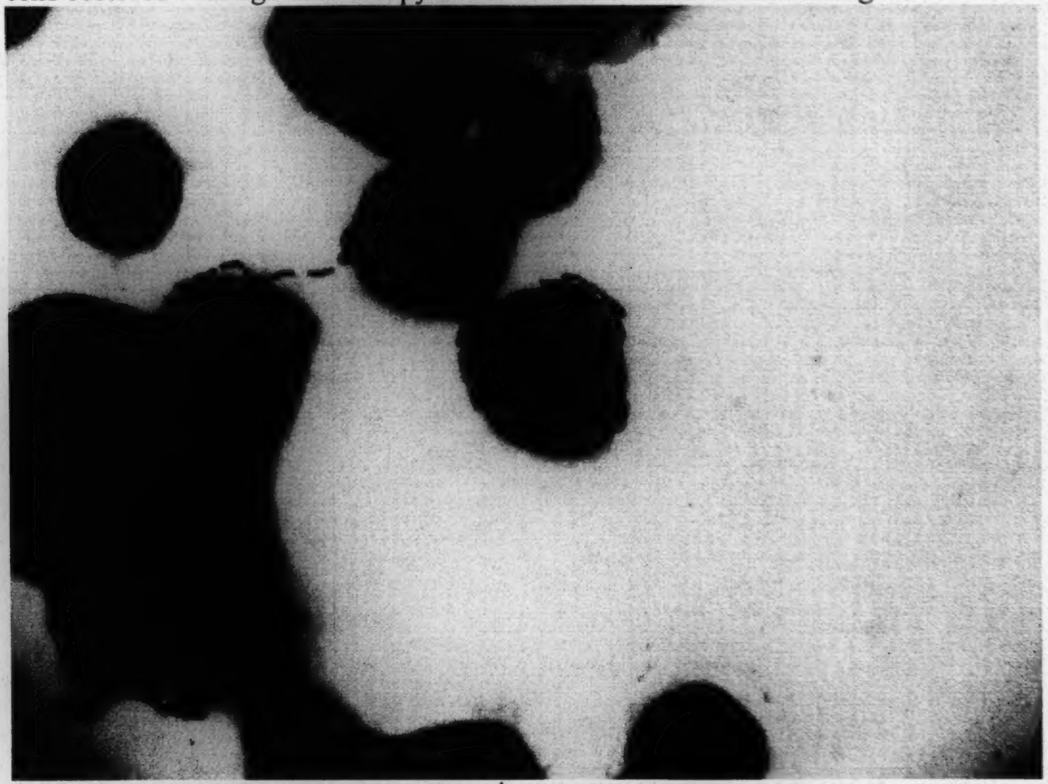
$^{\Psi}$ Chemical or Physical Treatment: Average number of adhering lactobacilli in 20 microscopic fields ± standard deviation (minimum n=5); Determination of Adhesion Mechanism: Means in the same row with different letters are significantly ($p \leq 0.05$) different) thus the treatment affected adhesion, signifying it's role in adhesion. Control studies were performed in the absence of chemical or physical treatment. N/D: No data.

Figure 3.1. Adhesion of *Lactobacillus casei* subsp. *casei* CSCC 2601 to HT 29 colon cancer cells observed with light microcopy and oil immersion after Gram staining.



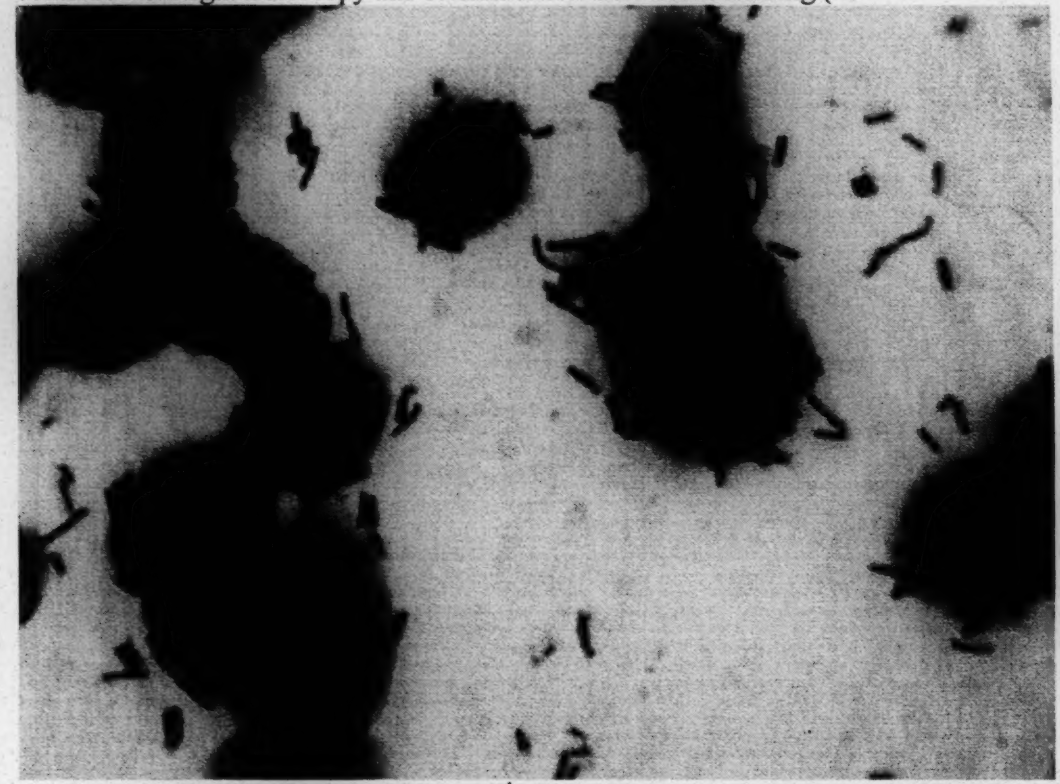
HT29 colon cancer cells were seeded (6.3 × 10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS to remove all nonadherent bacteria. The monolayers were fixed with methanol (3mL/well) for 5 minutes and then Gram stained. The cells were then examined by light microscopy under oil immersion. Each experiment was performed three times in triplicate and for each sample, 20 microscopic fields were counted.

Figure 3.2. Adhesion of *Lactobacillus acidophilus* NRC 13017 to HT29 colon cancer cells observed with light microscopy and oil immersion after Gram staining.



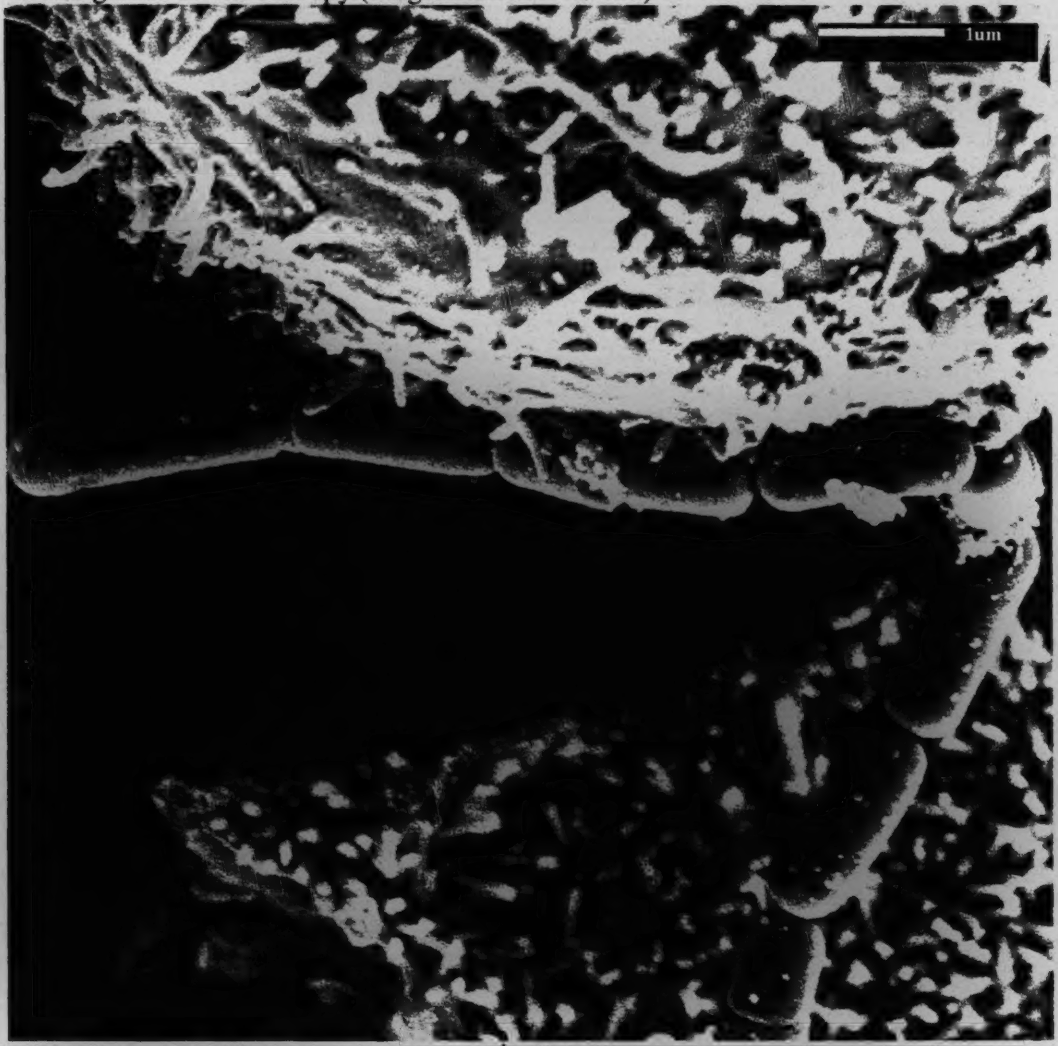
HT29 colon cancer cells were seeded (6.3 × 10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS to remove all nonadherent bacteria. The monolayers were fixed with methanol (3mL/well) for 5 minutes and then Gram stained. The cells were then examined by light microscopy under oil immersion. Each experiment was performed three times in triplicate and for each sample, 20 microscopic fields were counted.

Figure 3.3 Adhesion of *Lactobacillus acidophilus* MR100 to HT 29 colon cancer cells observed with light microcopy and oil immersion after Gram staining (×.



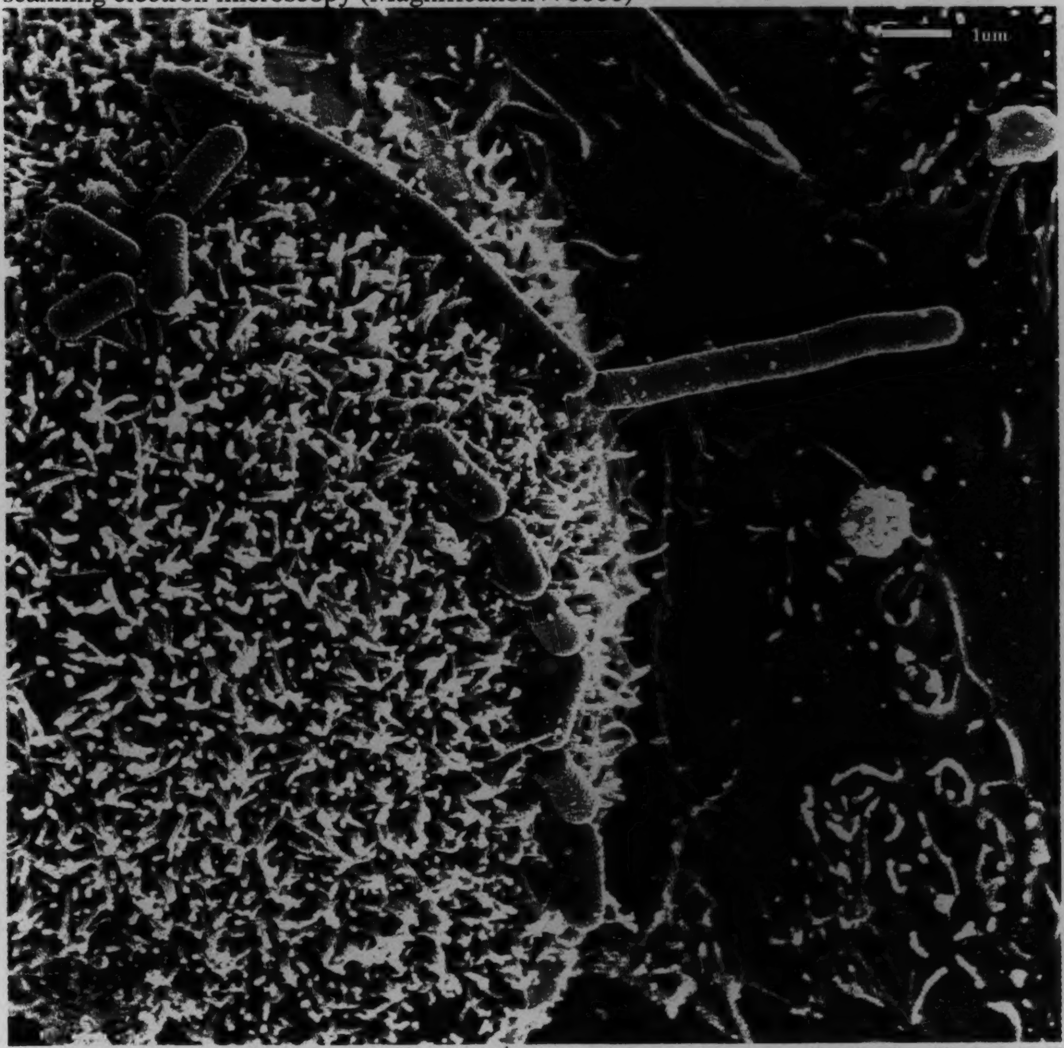
HT29 colon cancer cells were seeded (6.3 × 10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS to remove all nonadherent bacteria. The monolayers were then fixed with methanol (3mL/well) for 5 minutes and then Gram stained. The cells were then examined by light microscopy under oil immersion. Each experiment was performed three times in triplicate and for each sample, 20 microscopic fields were counted.

Figure 3.4 Adhesion of *Lactobacillus casei* subsp. *casei* CSCC 2601 observed through scanning electron microscopy (Magnification × 10 000)



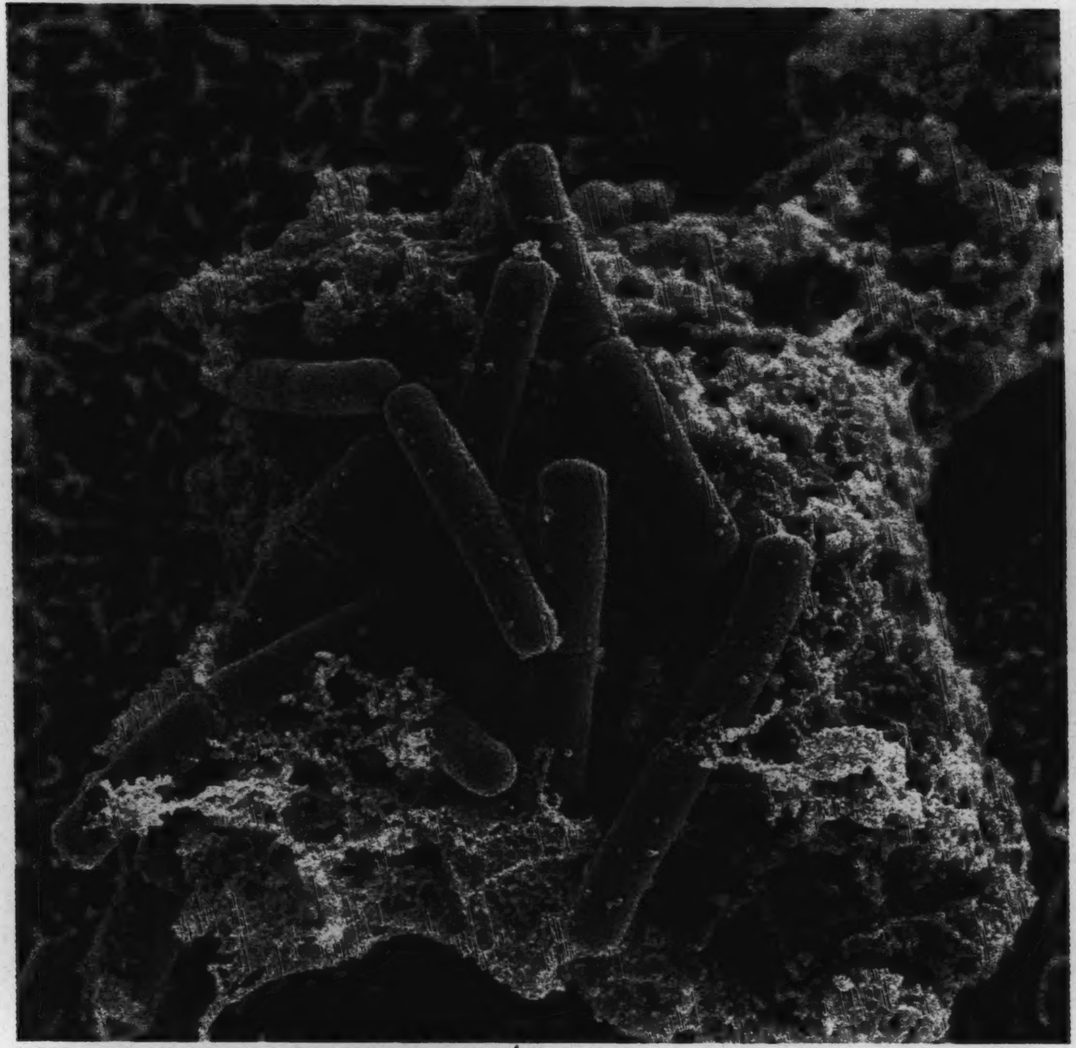
HT29 colon cancer cells were seeded (6.3 × 10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS to remove all nonadherent bacteria. The cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at room temperature for 1 hour. The cells were then washed with phosphate buffer to remove residual glutaraldehyde. The cells were then post fixed for 30 minutes at 37°C with 2% (w/v) osmium tetraoxide in 1M phosphate buffer. The cells were washed with phosphate buffer. The cells were then dehydrated in a graded series of ethanol (30, 50, 70, 80, 90 98% v/v) and then passaged through a graded series of amyl acetate (50, 70, 90, 100% v/v in ethanol). The cells were then dried in a critical point dryer (Polaron Jumbo), coated with gold (SEMprep 2, Nanotech) and examined via SEM (JEOL JSM 6301FXV).

Figure 3.5 Adhesion of *Lactobacillus casei* subsp. *casei* CSCC 2601 observed through scanning electron microscopy (Magnification × 6000)



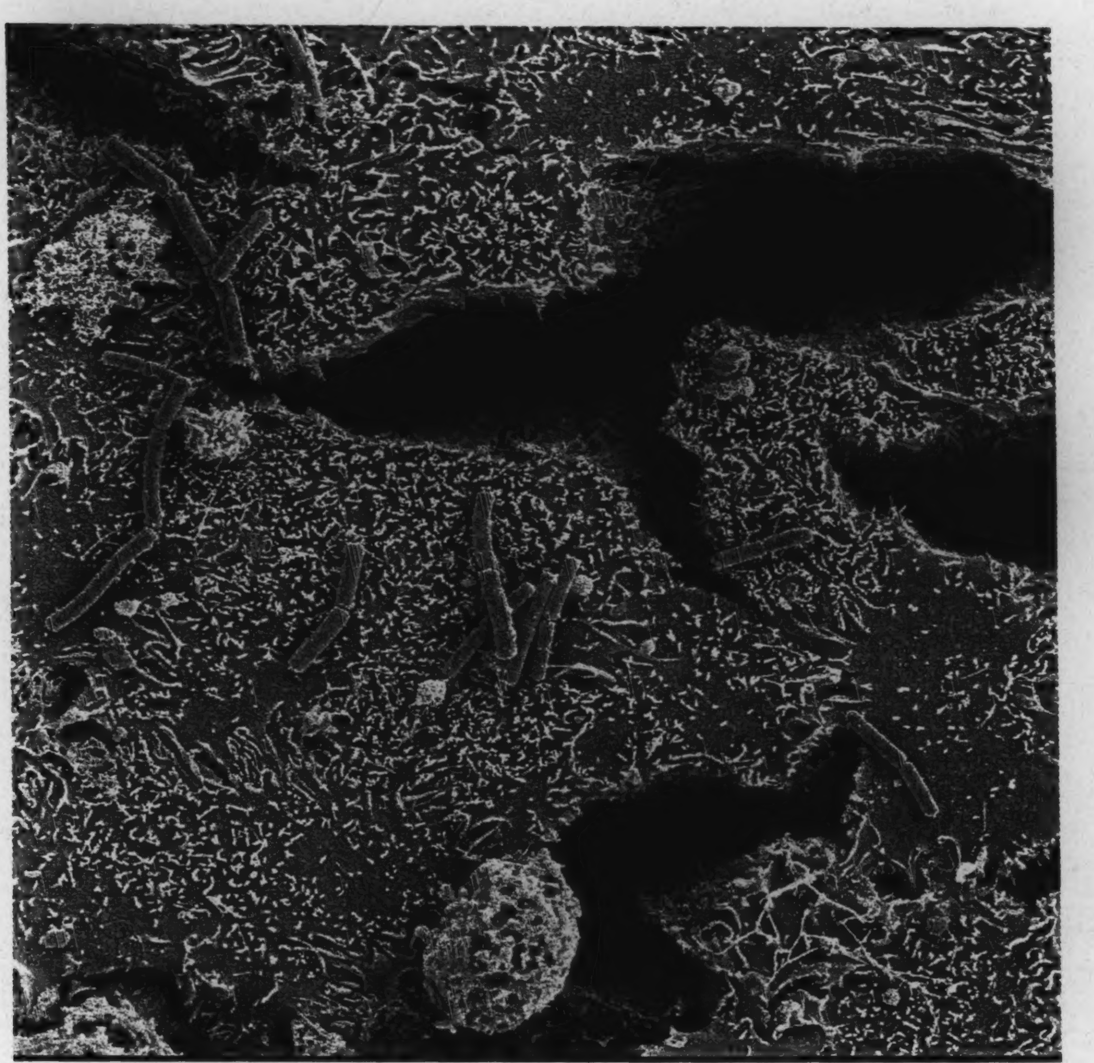
HT29 colon cancer cells were seeded (6.3×10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS to removal all nonadherent bacteria. The cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at room temperature for 1 hour. The cells were then washed with phosphate buffer to remove residual glutaraldehyde. The cells were then post fixed for 30 minutes at 37°C with 2% (w/v) osmium tetraoxide in 1M phosphate buffer. The cells were washed with phosphate buffer. The cells were then dehydrated in a graded series of ethanol (30, 50, 70, 80, 90 98% v/v) and then passaged through a graded series of amyl acetate (50, 70, 90, 100% v/v in ethanol). The cells were then dried in a critical point dryer (Polaron Jumbo), coated with gold (SEMprep 2, Nanotech) and examined via SEM (JEOL JSM 6301FXV).

Figure 3.6 Adhesion of *Lactobacillus bulgaricus* MR110 observed through scanning electron microscopy (Magnification × 9500)



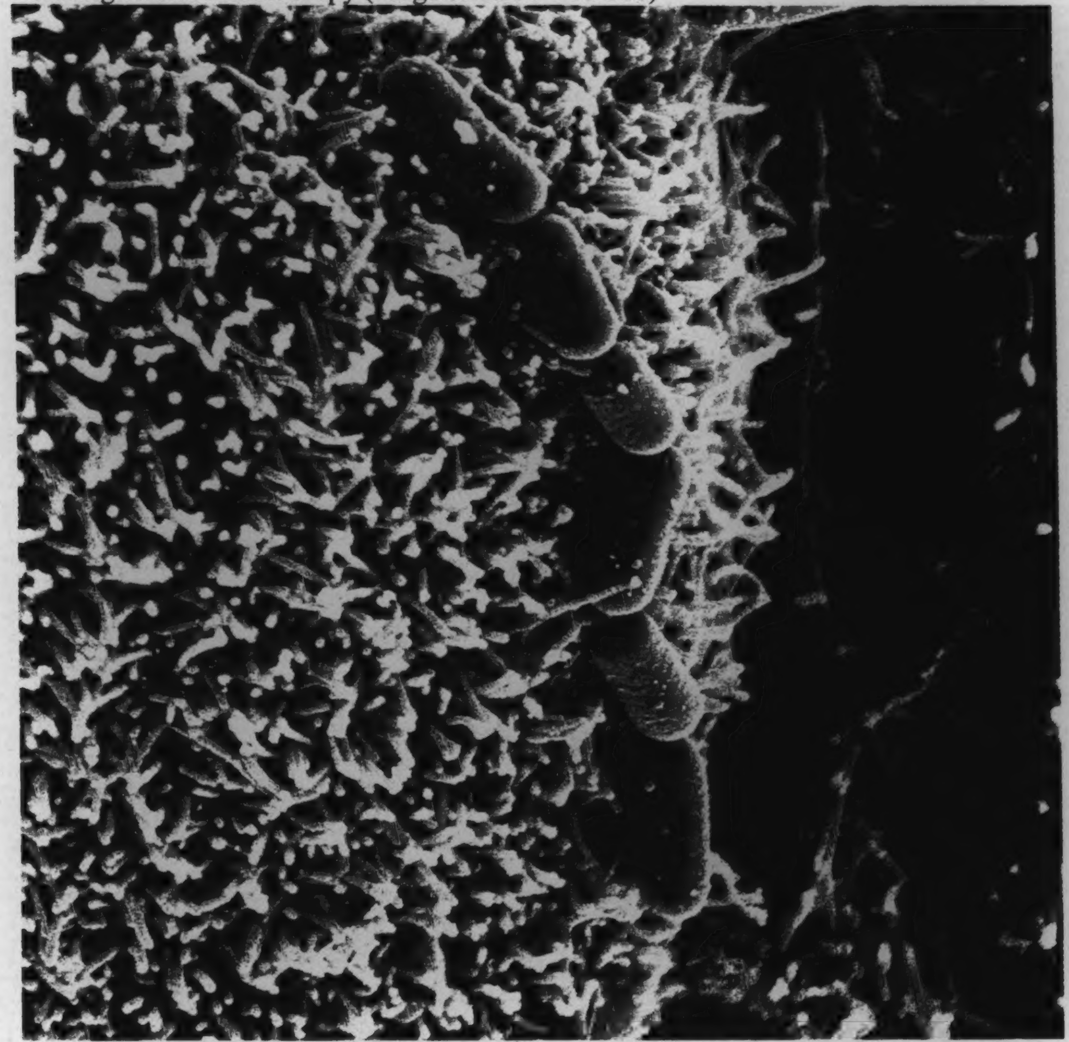
HT29 colon cancer cells were seeded (6.3×10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS tó removal all nonadherent bacteria. The cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at room temperature for 1 hour. The cells were then washed with phosphate buffer to remove residual glutaraldehyde. The cells were then post fixed for 30 minutes at 37°C with 2% (w/v) osmium tetraoxide in 1M phosphate buffer. The cells were washed with phosphate buffer. The cells were then dehydrated in a graded series of ethanol (30, 50, 70, 80, 90 98% v/v) and then passaged through a graded series of amyl acetate (50, 70, 90, 100% v/v in ethanol). The cells were then dried in a critical point dryer (Polaron Jumbo), coated with gold (SEMprep 2, Nanotech) and examined via SEM (JEOL JSM 6301FXV).

Figure 3.7 Adhesion of *Lactobacillus bulgaricus* MR110 observed through scanning electron microscopy (Magnification × 2500)



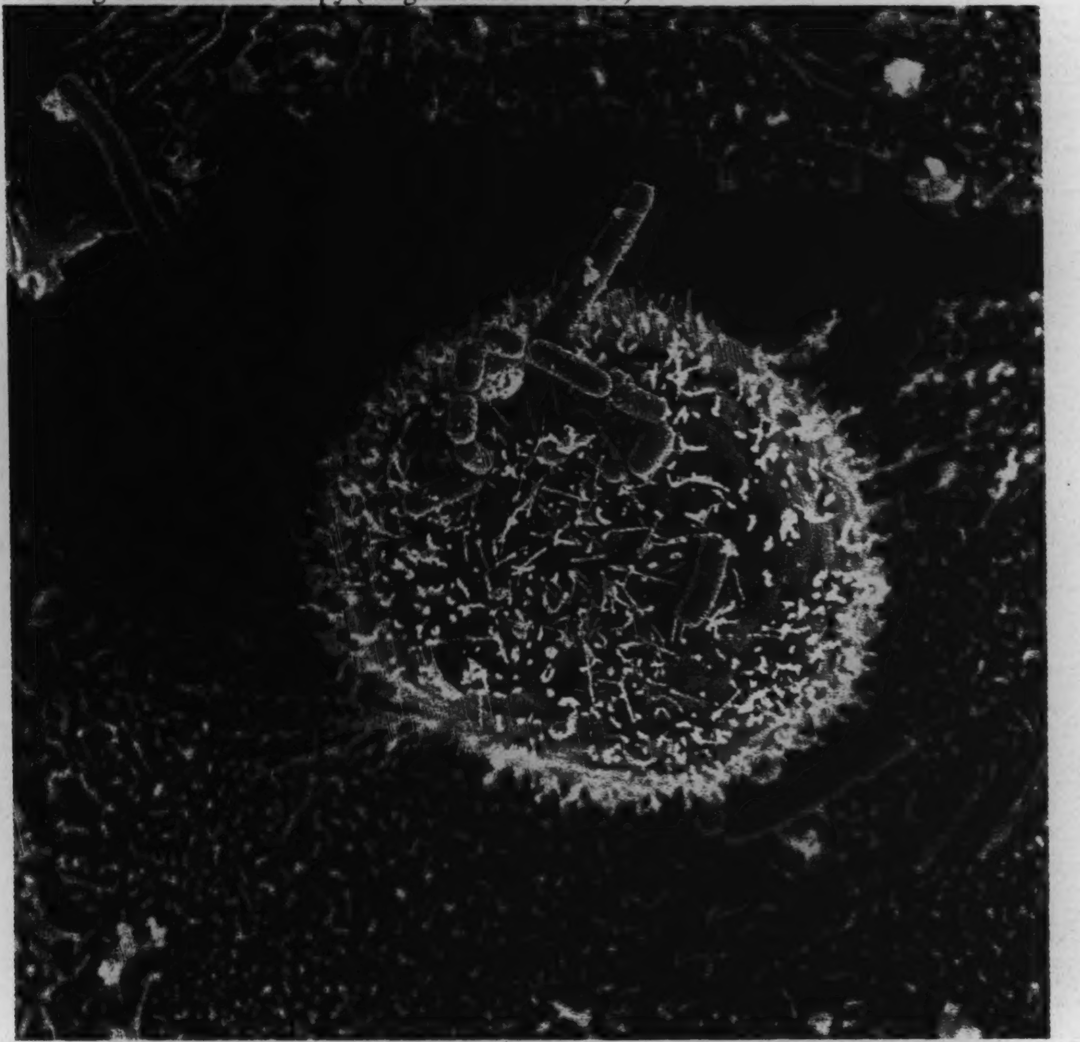
HT29 colon cancer cells were seeded (6.3 × 10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS to removal all nonadherent bacteria. The cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at room temperature for 1 hour. The cells were then washed with phosphate buffer to remove residual glutaraldehyde. The cells were then post fixed for 30 minutes at 37°C with 2% (w/v) osmium tetraoxide in 1M phosphate buffer. The cells were washed with phosphate buffer. The cells were then dehydrated in a graded series of ethanol (30, 50, 70, 80, 90 98% v/v) and then passaged through a graded series of amyl acetate (50, 70, 90, 100% v/v in ethanol). The cells were then dried in a critical point dryer (Polaron Jumbo), coated with gold (SEMprep 2, Nanotech) and examined via SEM (JEOL JSM 6301FXV).

Figure 3.8 Adhesion of *Lactobacillus casei* subsp. *casei* CSCC 2601 observed through scanning electron microscopy (Magnification × 10 000)



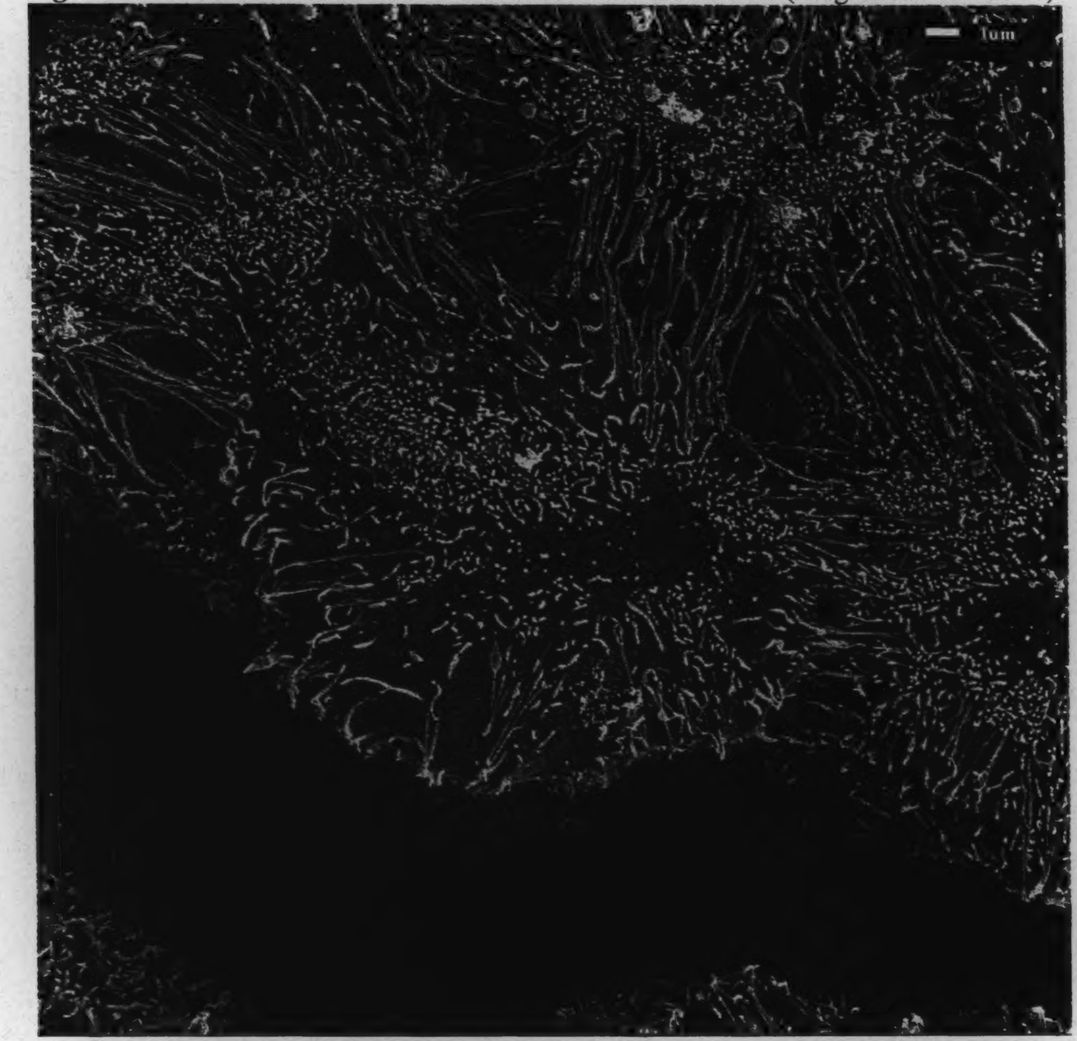
HT29 colon cancer cells were seeded (6.3×10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS to removal all nonadherent bacteria. The cells were then fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at room temperature for 1 hour. The cells were then washed with phosphate buffer to remove residual glutaraldehyde. The cells were then post fixed for 30 minutes at 37°C with 2% (w/v) osmium tetraoxide in 1M phosphate buffer. The cells were washed with phosphate buffer. The cells were then dehydrated in a graded series of ethanol (30, 50, 70, 80, 90 98% v/v) and then passaged through a graded series of amyl acetate (50, 70, 90, 100% v/v in ethanol). The cells were then dried in a critical point dryer (Polaron Jumbo), coated with gold (SEMprep 2, Nanotech) and examined via SEM (JEOL JSM 6301FXV).

Figure 3.9 Adhesion of *Lactobacillus casei* subsp. *casei* CSCC 2601 observed through scanning electron microscopy (Magnification × 4 000)



HT29 colon cancer cells were seeded (6.3×10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). Overnight cultures of lactic acid bacteria were added to each well containing HT29 cells. The plates were incubated for 1 hour (37°C in 5% CO_2/95% air incubator). The monolayers/bacteria were then washed with PBS to removal all nonadherent bacteria. The cells were then fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at room temperature for 1 hour. The cells were then washed with phosphate buffer to remove residual glutaraldehyde. The cells were then post fixed for 30 minutes at 37°C with 2% (w/v) osmium tetraoxide in 1M phosphate buffer. The cells were washed with phosphate buffer. The cells were then dehydrated in a graded series of ethanol (30, 50, 70, 80, 90 98% v/v) and then passaged through a graded series of amyl acetate (50, 70, 90, 100% v/v in ethanol). The cells were then dried in a critical point dryer (Polaron Jumbo), coated with gold (SEMprep 2, Nanotech) and examined via SEM (JEOL JSM 6301FXV).

Figure 3.10 HT29 Human colon cancer cell line without bacteria (Magnification × 2500)



HT29 colon cancer cells were seeded (6.3×10^4 cells/well) onto glass coverslips in 6 well plates and incubated overnight (37°C in 5% CO_2/95% air). The monlayer was then fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at room temperature for 1 hour. The monolayer was then washed with phosphate buffer to remove residual glutaraldehyde. The monolayer was then post fixed for 30 minutes at 37°C with 2% (w/v) osmium tetraoxide in 1M phosphate buffer. The monolayer was washed with phosphate buffer. The monolayer was then dehydrated in a graded series of ethanol (30, 50, 70, 80, 90 98% v/v) and then passaged through a graded series of amyl acetate (50, 70, 90, 100% v/v in ethanol). The monolayer was then dried in a critical point dryer (Polaron Jumbo), coated with gold (SEMprep 2, Nanotech) and examined via SEM (JEOL JSM 6301FXV).

CHAPTER 4[*]

The Inhibition or Stimulation of Inducible Nitric Oxide Synthase (iNOS) in Raw 264.7 Mouse Macrophage Cell Line by Dairy Derived LAB and Milk Components

4.1 INTRODUCTION

Nitric oxide (NO) is a pleiotropic free radical messenger that is produced in the body via the action of nitric oxide synthase on the substrate L-arginine. Nitric Oxide has been reported to affect cellular permeability (Menconi et al., 1998), have vasodilatory functions (Lippe and Holzer, 1992) and many other physiological functions (Brown et al., 1993; Pique et al., 1989; Holzer and Sametz, 1986; Holzer et al., 1990; Kanwar et al., 1994; Obermeier et al., 1999a, b; Huang et al., 1998).

There are three forms that are present in the body, two that are constitutively produced and one that is inducible. The two that are constitutively produced are regulated at the enzymatic level by calcium and calmoduline binding. These two forms, neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase, present in the nervous system and the vascular endothelium, respectively, produce nitric oxide at low levels that result in physiologic effects such as relaxation of vascular smooth muscle and neurotransmission. The third form, inducible nitric oxide synthase (iNOS) is produced in response to microbes, cytokines and other stimuli in immune cells (Cross and Wilson, 2003). Inducible nitric oxide synthase is pleiotropic in nature. It has strong potential to induce either proinflammatory or anti-inflammatory effects. However, whether NO is beneficial or deleterious still remains controversial. It is thought that at low levels of NO, such as that produced by the constitutive forms, NO confers a protective effect; whereas the levels that are produced by iNOS (micromolar), NO is likely to be proinflammatory and deleterious to healthy tissue (Cross and Wilson, 2003).

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Over the last decade, NO oxide has been researched extensively for its role in the pathology of inflammatory bowel disease (IBD); however, its effect is still not completely understood. Scientists found that in patients with active colitis there are increased levels of citrulline, a metabolite produced from arginine in equimolar concentrations when NO is liberated by iNOS (Middleton et al., 1993). Patients with ulcerative colitis have 8-100 fold higher iNOS activity than in normal controls (Boughton-Smith et al., 1996; Lundberg et al., 1994). Children with IBD and cystic fibrosis have elevated levels of iNOS (Canini et al., 2002; Ljung et al., 2001). These studies lead researchers to speculate that the elevated NO levels may contribute to the etiology of IBD. In fact, research using synthetic inhibitors of iNOS has shown success in the treatment of IBD associated inflammation. NG-nitro-L-arginine methylester (L-NAME) and aminoguanidine (AG) (Grisham et al., 1994; Rachmilewitz et al., 1995a; Rachmilewitz et al., 1995 b) significantly decreased inflammation scores and decreased NO production in rats. However, this effect seems to be dependent upon the stage of activation the disease is in (Kiss et al., 1997)

Chronic inflammation can lead to the development of at least one third of all human cancers (Hussain et al., 2003; Harris, 1994; Asaka et al., 1997). Reactive oxygen or nitrogen species are released as a result of pathogenic insult or tissue injury; however, in chronic inflammatory conditions, where there is sustained release, damage occurs to DNA repair proteins and ultimately damage to DNA itself (Hussain, 2003). Thus, chronic inflammation in IBD is associated with increased incidence of colon cancer (Munkholm, 2003; Gillen et al., 1994). Furthermore, studies have demonstrated that there is not a genetic component involved in IBD associated development of colon cancer (Riegler et al., 1998; Askling et al., 2001).

In cancer NO plays a contradictory role. NO induced DNA alterations can lead to p53 tumour suppressor activation thus contributing to positive regulation of cellular proliferation, regulation of cell cycle check points, and ultimately to anticarcinogenic effects of p53 (Messmer and Brune, 1996; Wink et al., 1998). Paradoxically, at higher concentrations NO has mutagenic effects on p53, leading to accumulation of mutant p53 and ultimately malignancy (Greenblatt et al., 1994). Indeed, in precancerous lesions of inflamed ulcerative colitis tissue there is increased iNOS expression and p53 mutation (Goodman et al., 2004) and others have found that iNOS is frequently expressed in 60% of human adenomas and 20-25% of colon carcinomas as compared to normal surrounding tissue (Ambs et al., 1998).

Thus it seems that NO levels/iNOS activity in the body is tightly regulated; in some disease states, this tightly regulated system of NO is derailed. For this reason, it seems logical that NO is a therapeutic target in disease resolution. Macrophages are key immune modulators against infection, inflammation and tumour development. The production of NO by macrophages is an integral component of the body's antipathogenic and tumouridical response (Nathan and Xie, 1994; Rubbo et al., 1994). NO production by macrophages is predominantly by the inducible form of NOS (Kroncke et al., 1995).

Dairy derived LAB and bioactive peptides have been found to have anticancer and anti-inflammatory properties in both cancer and IBD (Brady et al., 2000; Varcoe et al., 2003). The antitumour activities have generally been ascribed to binding of mutagenic/carcinogenic compounds, inhibiting noxious metabolite formation, protection against DNA demethylation (McIntosh et al., 1998), or altering deleterious microflora (Day et al., 2004). Recently, the focus has shifted to immunostimulatory or immunomodulatory effects of dairy derived LAB and proteins. As such NO is a powerful immune mediator within the body, slight dysregulation can result in catastrophic results such as chronic inflammation and cancer.

The purpose of this study was to identify whether dairy derived LAB and milk proteins can have immunomodulatory potential through altering NO secretion by *Salmonella typhimurium* LPS stimulated or unstimulated mouse macrophages. In addition, the focus was on identification of components that exerted the effects. The results from this study could identify possible compounds that could be used as immunoadjuvants in disease prevention and/or treatment

4.2 MATERIALS AND METHODS

4.2.1 Raw 264.7 Murine Macrophage Cell Line Propagation and Growth

A murine macrophage cell line RAW 264.7 (ATCC: TIB-71) cells were kindly provided by Luis Hildalgo from Dr. Halloran's laboratory (Medical Microbiology and Immunology at the University of Alberta, Edmonton, Alberta, Canada). RAW 264.7 cells were grown according to American Type Culture Collection (ATCC) recommendations. Briefly, RAW 264.7 cells were grown in Dulbecco's Minimal Essential Media (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1.5 g/L of sodium bicarbonate (Sigma-Aldrich), 10% (v/v) FCS (Gibco Laboratories, Chagrin Falls, IL, USA), 100 U/mL penicillin, 100μg/mL streptomycin and 100 U/mL amphotericin (Sigma-Aldrich). Cells were grown to 80% confluence at 37° C in 5% CO_2/95% air in 75 cm^2 vented tissue culture flasks (Corning, Fisher Scientific, Nepean, Ontario, Canada). Cell number and viability were assessed using trypan blue exclusion with a haemocytometer. DMEM was changed every second day to third day. Cell detachment was achieved via trypsinization (Sigma-Aldrich) or a cell scraper.

RAW 264.7 murine macrophage cells were seeded at a concentration of 1×10^6 cells/well in a 96 well plate (Corning). These cells were allowed to attach for 24 hours. The cells were washed 5 times with fresh DMEM (without antibiotic/antimycotic) to remove all residual amounts of antibiotic. Then 100 uL of *Salmonella typhimurium* LPS (1 ug/mL final concentration; Sigma Aldrich) dissolved in DMEM was added to each well to activate the macrophages. Then 100 uL of the bacterial samples were added to each well. *Salmonella typhimurium* LPS was used as a positive control (100 μL/well). The plates were then incubated at 37°C in 5% CO_2 for 48 hours. After incubation, the plates were centrifuged at 3000 rpm (AccuSpin Micro, Fisher Scientific) for 10 minutes (Ring et al., 2000) and the supernatants collected were used immediately for nitric oxide assay. Each assay was performed three times in triplicate.

For unstimulated NO testing, the procedure was repeated as described above, however, LPS was only added to the positive control wells. The other wells contained treatment samples only. Each assay was performed three times in triplicate.

4.2.2 Probiotic LAB Propagation and Growth

Eight strains of LAB (*Lactobacillus acidophilus* MR 100 (MR 100), *Lactobacillus bulgaricus* MR 110 (MR 110), *Lactobacillus acidophilus* NRC 13017 (NRC 13017), *L.acidophilus* NRC 13019 (NRC 13019), *Lactobacillus casei* ADA 03 (ADA 03), *Lactobacillus casei* NRC 13005 (NRC 13005), *Lactobacillus casei* subsp. *casei* CSCC 2601 (CSCC 2601) and Yogurt Culture YC 085 (YC 085)) were used in this assay. These strains were grown in MRS broth (Difco) at 37°C for 24 hours (bacteria cell density 1×10^9 cells/mL). The cells were washed three times with PBS. After each wash, cell suspension was centrifuged at 10,000 g for 2 minutes to collect cells. A portion of bacterial cells of each strain were then resuspended into DMEM (10% Fetal Calf Serum) without antibiotic/antimycotic immediately prior to addition to cell culture. The remaining portion of bacterial cells of each strain was stored at -70°C in MRS broth containing 20% glycerol (Sigma Aldrich).

The previously mentioned eight strains of LAB were heat killed. The cells were washed three times with PBS, centrifuged at 10 000 g for 2 minutes to remove all MRS broth residue and resuspended in PBS. The cells were then placed into a 60°C water bath and incubated for 60 minutes. The heat-killed cells were then centrifuged (10 000 g for 2 minutes), resuspended into DMEM (10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin and 100 U/mL of amphotericin), and stored at –20°C until used.

4.2.3 *L. casei ADA 03 Peptidoglycan Isolation and Purification*

Peptidoglycan was isolated from *L.casei* ADA 03 according to the methods reported by de Ambrosini et al., (1996). Briefly, resuspended cells of *L.casei* ADA 03 in PBS were subjected to mechanical disruption using a French Press. Broken cells were centrifuged (10 000 g) for 10 minutes and the pellet obtained was suspended in 4% boiling SDS (sodium dodecyl sulfate; Sigma Aldrich) in order to dissolve the cell wall fraction. After 18 hours of incubation, the suspension was centrifuged at 20, 000 g for 20 minutes, and washed with PBS to remove SDS. The pellet was then treated with RNase and DNase to remove nucleic acids, followed by washing with PBS. The pellet suspended in PBS was then treated with trypsin to digest any cell wall associated proteins. This preparation of cells was then washed with PBS and treated with 2% SDS for 4 hours to remove all proteins. Then the sample was washed thoroughly with PBS and deionized water (Milli Q, Millipore). This fraction was considered as the whole cell wall preparation.

The insoluble cell wall precipitate was then treated with 10% trichloroacetic acid (Sigma Aldrich) for overnight period to remove teichoic acid from in the cell wall. The suspension was centrifuged and the pellet was washed thoroughly with PBS and MilliQ water. This pellet was taken as peptidoglycan. For experimental testing, the pellet was suspended in McCoys Modified 5A media.

4.2.4 Isolation of Fermented Milk Supernatant

A 1% (v/v) inoculum of the previously mentioned LAB were inoculated into 12% (w/v) skim milk containing 1% (w/v) glucose and 1% (w/v) yeast and incubated for 24 or 48 hours. The fermented milk samples were centrifuged at 10 000 g for 2 minutes. The collected supernatant was filtered with a syringe filter (0.25 μm pore size; Acrodisc, Gelman Sciences, Ann Arbor, MI, U.S.A). Aliquots of the supernatants were stored at -20 °C until required for experiments.

4.2.5 Milk Derived Protein/Peptide Preparation

Sodium caseinate, β-lactoglobulin, lactoferrin, α-lactalbumin, casein hydrolysate and β-casomorphin-7 were all purchased from Sigma Aldrich (St. Louis, MO, USA). The following concentrations were used for each protein/peptide fraction: sodium caseinate: 75, 37.5 and 18.75 mg/mL; β-lactoglobulin: 120, 90, 60, 30, and 15 mg/mL; lactoferrin: 500, 200, 100, 20 ug/mL; α-lactalbumin: 35, 25 and 10 μg/mL; casein hydrolysate: 40, 20 and 10 mg/mL and β-casomorphin-7: 125, 62.5 and 31.25 μg/mL. Each protein/peptide solution was made in DMEM supplemented with 1.5 g/L of sodium bicarbonate and 10% FCS and filter sterilized with a 0.25 μm syringe filter disc (Acrodisc, Gelman Sciences). The sterile solutions were made immediately prior to nitric oxide testing.

4.2.6 Nitric Oxide Assay (Griess Reaction)

Nitric oxide production was measured using the Griess reaction (Green et al., 1982). Briefly, immediately prior to assay equal amounts of N-(1-naphthyl)ethylenediamine dihydrochloride (100 mg dissolved into 100 mL distilled water; Sigma-Aldrich) and sulfanilamide (1g in 100 mL of 5% phosphoric acid; Sigma Aldrich) were combined together to create the Griess reagent. Sodium nitrite (2 mM stock solution, diluted in DMEM) (Sigma) was used as standards, plated at concentrations from 0-200 μM in triplicate.

In an optically clear 96 well flat bottom plate (Corning), containing 100 μL of the Griess Reagent was added 100 uL of nitrite free control, unknown samples or nitrite standard. The plates were then incubated at room temperature for 30 minutes to allow development and stabilization of the chromophore. Absorbance was measured at 550 nm using a Microplate reader (SpectraMax 190, Molecular Devices Corporation, Sunny Vale, California, U.S.A).

4.2.7 Statistical Analysis

Statistical Analysis was carried out in the Statistica program (Statistica 6.0). The data was analyzed through analysis of variance, Tukey's test, correlation of analysis and regression analysis.

4.3 RESULTS AND DISCUSSION

NO is a free radical messenger that is intricately involved in the progression or resolution of many disease states. iNOS is responsible for producing physiologically relevant concentration of NO in the body. NO activity is paradoxical, depending upon concentration, which disease or at what stage in the disease, NO can have extremely powerful yet opposite effects. Upregulation of iNOS activity and thus nitric oxide production contributes to inflammation and oxidative stress (Cross and Wilson, 2003). This inflammatory response is the result of NO reacting with superoxide leading to the formation of peroxynitrite anion, a highly cytotoxic oxidant (Beckman and Koppenol, 1996). In cancer upregulation of NO, can induce neoplastic transformation in mouse fibroblasts (Mordan et al., 1993), or induce secretion of mucin by colonic adenocarcinoma cells, which confers a protective effect to cancer cells (Gottke and Chadee, 1996). Indeed iNOS is expressed in 60% of human adenocarcinoma cells, whereas there is either very low or no expression in normal tissue (Ambs et al., 1998).

Conversely, NO provides protective functions against pathogenic organisms (Goodman et al., 2004); in Alzheimer's patients, treatment with NO mimetics circumvents dementia (Thatcher et al., 2005). Furthermore, NO acts as a potent vasodilator (Lippe and Holzer, 1992), plays an integral role in cellular permeability (Menconi et al., 1988) and many other functions.

In cancer, the role of NO is equally as pleiotropic. At low concentrations, NO is involved in activating tumour suppressor gene p53 (Goodman et al., 2003; Hussain et al., 2003), yet at the same time exhibiting protumour attributes such as antiapoptotic effects (Zhao et al., 1998) and stimulation of tumour angiogenesis (Fukumura and Jain, 1998). Furthermore, at higher concentrations, NO can induce mutations in tumour suppressor gene p53 (Goodman et al., 2003; Hussain et al., 2003) yet at the same time induce apoptosis and arresting tumour cell growth (Xie and Fidler, 1998; Kwak et al., 2000).

The role of NO in the pathology of IBD is still contentious. Many researchers have demonstrated the positive effects of nitric oxide inhibition in the attenuation of the disease state (Rachmilewitz et al., 1995; Kankuri et al., 2001), however, others have not found a beneficial role for NO. McCafferty et al. (1999), found in iNOS deficient mice, there is a higher level of colonic inflammation 72 hours after administration of trinitrobenzene sulfonic acid (TNBS), as compared to wild type mice, suggesting the possible protective role of NO in intestinal inflammation. Indeed, the addition of the anti-inflammatory compound mesalamine that has been modified to carry NO had greater anti-inflammatory effect than mesalamine alone. The researchers have attributed this beneficial effect to nitric oxide (Wallace et al., 1999).

Regardless of the conflicting physiological role of NO in disease progression, the search for compounds that can be used to manipulate NO stimulation or inhibition by immune cells is still being extensively explored. LAB and milk derived proteins have been found to alter the body's immunologic response (as reviewed by Dugas et al., 1999). For this reason we examined the effect various strains of dairy derived LAB and milk proteins on nitric oxide production and inhibition in RAW 264.7 mouse macrophage cell line.

4.3.1 The Effect of Dairy Derived Milk Proteins and Bacteria on Unstimulated RAW 264.7 Mouse Macrophage Nitric Oxide Production

Interestingly, for the bacterial treatments (Table 4.1), all the heat killed cells and peptidoglycan were able to elicit a response. In the LPS stimulated control, 31.89 (±0.30) µg/mL of NO was produced. Our heat killed yogurt culture YC085 cells elicited significant a response almost as high (27.15 µg/mL). Although, not as strong of a response was induced by mixture, NRC 13019, NRC 13005, MR 100 and ADA 03 peptidoglycan they induced a statistically significant 32% to 63% as much NO as the control. The rest of the heat killed cells (NRC 13017, L. casei ADA 03, MR 110 and CSCC 2601), also induced mouse macrophages to produce NO, although, their effect was mild, concentrations were still statistically significant compared to the negative control. It is interesting to note, that peptidoglycan isolated from ADA 03 had a stronger stimulatory effect than the live cells, leading us to possibly speculate that the active component for ADA 03 is in the peptidoglycan. We cannot completely conclude this, because of the high concentration of peptidoglycan we used as compared to the actual cells. However, the concentrations used in this experiment are similar to that of Fichera and Giese (1994). Unfortunately, none of the live cells nor the fermented milk supernatants elicited any NO from the unstimulated macrophage cells, leading us to speculate that some component within the live cells is preventing NO production or interaction with the macrophage cell. Perhaps during heat killing, some heat labile cell surface component(s) are modified to render it active. However, further research needs to be done before any firm conclusions can be drawn. In addition, we can rule out that some NO production stimulating compound is released into the spent supernatant during fermentation.

Our results are in line with that of various researchers. Tejada-Simon and Pestka (1998) found that several strains of heat killed cells and peptidoglycan isolated from LAB were able to stimulate NO production in unstimulated RAW 264.7 cells, reaching similar concentrations to the ones exhibited by LPS stimulation. Korhonen and colleagues

(2001) found that heat killed *L. rhamnosus* GG (LGG) (form commonly used in dairy products) significantly increased nitrite accumulation in culture medium by J774 murine mouse macrophages, however, they also found that viable and lyophilized forms induced NO production, a characteristic none of our live strains demonstrated. This was found to be concurrent with upregulation of iNOS transcription, as detected by the expression of the iNOS gene. These researchers suggest that lipoteichoic acid, found in the cell wall of LGG may be the active component (Korhonen et al., 2001), which are reflected by our ADA 03 peptidoglycan results. Commercial and human isolates of *Bifidobacterium* (Park, et al., 1999) and kimchi bacterial isolates, *Leuconostic mesenteroides* and *Lactobacillus plantarum,* (Hur et la., 2004) stimulated the production of NO from RAW 264.7 macrophages. In animal studies, oral administration of *Lactobacillus farciminis* improved experimentally induced colitis; the researchers attributed the effect to NO (Lamine et al., 2004).

The effects of heat killed LAB and cell wall components have been attributed to several mechanisms. It is thought that through the stimulation of macrophages, potent nitric oxide inducing cytokine tumour necrosis factor (TNF) is produced, resulting in increased production of NO (Tejada- Simon and Pestka, 1999; Zidek et al., 1998; Park et al., 1999). Indeed, in other experiments performed in this laboratory, heat killed cells were able to induce very mild secretion of TNF from unstimulated mouse macrophage cells. Perhaps, this low level of secretion is enough to stimulate the production of NO from mouse macrophages.

Others suggest that LGG stimulatory activity is mediated by cell surface receptor CD14 and toll like receptor (TLR). TLR together with CD14 leads to the activation of inflammatory transcription factor nuclear factor KB (NF-KB). There are NF-KB promoter regions within the iNOS gene, inhibitors of NF-KB activation, prevent NO production and iNOS protein expression. PDTC, an inhibitor of NF-KB activation, blocked all NO produced from J774 cells after LGG stimulation, suggesting NF-KB activation as a mechanism of action (Korhonen, et al., 2002). Tyrosine kinase is involved in signal transduction of LPS response in macrophages and is involved in regulating induction of iNOS and NO following inflammatory stimulus (Geng et al., 1995). The addition of tyrosine kinase inhibitors to LGG stimulated J774 mouse macrophages completely abrogates all activity (Korhonen, 2002), suggesting the role of tyrosine kinase in signal transduction of NO production by LGG. Others have suggested that the toxic effects of NO are attributed to iron status in cells. NO mediates iron loss from cells; this loss of iron inactivates many enzymatic processes within the citric acid cycle that are dependent upon iron as a cofactor in energy generation (Drapier and Hibbs, 1988; Henry et al., 1993; Klimp et al., 2002). Furthermore, NO also has the ability to alter activity of ion channels in cells, thus disrupting mitochondrial membrane potential (Richter et al., 1994; Klimp et al., 2002).

Our results demonstrate that certain cell wall components may have therapeutic potential in the modulation of disease associated with decrease NO production. In addition, our results suggest that the long held belief that probiotics must be live to exert health benefits require re-evaluation. Heat killed cells of all eight of our bacterial strains had the ability to stimulate macrophage production of NO. Furthermore, peptidoglycan isolated from L. casei ADA 03 had significant NO inducibility. However, the fact that dead cells elicit a response does not come as a total surprise, since cell wall fractions from *Salmonella* and *E.coli* are powerful stimulators of immune cells *in vitro* (Alexander and Rietschel, 2001). However, not all probiotics are able to generate a similar response, *Bacillus fermus*, a nonpathogenic bacteria, was not able to stimulate NO production from mouse macrophages (Zidek et al., 1998).

Since the individual heat killed cells, had such significant stimulatory activity on NO secretion, we wanted to develop a cocktail of bacteria that could possibly have synergistic activity in NO stimulation (Table 4.1). We found that not only did the individual strains exert significant stimulatory activity, but the cocktail (mixture) of strains also had significant inhibitory activity upon NO secretion. However, the cocktail of heat killed bacteria had slightly lower activity than several of the individual strains, perhaps suggesting some bacterial strains had antagonistic activity against other strains. This is in agreement with other researchers. It has been shown that co-culture of different strains of bacteria result in inhibitory activity of one strain against another strain (Christensen, et al., 2002). Alternately, the observation that individual strains had a greater degree of stimulation than the cocktail perhaps is an indication that the individual strains are powerful enough stimulators. However, we cannot discount the effect of a highly concentrated mixed LAB cocktail, as seen in VSL#3, to have profound immunologic effects. Further experiments need to be performed.

Several of the milk proteins tested were also able to activate macrophages to produce NO. Casein hydrolysate, sodium caseinate and β-lactoglobulin all elicited a statistically significant NO response. Sodium caseinate (75mg/mL) produced the strongest response followed by β-lactoglobulin (60 and 30 mg/mL). Furthermore, sodium caseinate demonstrated a dose dependent response, with the highest degree of stimulation observed in the highest concentration tested. These milk proteins were able to stimulate between 4-28% NO as compared to the control. As compared to the bacteria, in general milk proteins were not able to elicit as strong of a response. These results also demonstrate that casein salt and hydrolyzed forms of casein are immunogenic and can induce NO production from unstimulated immune cells.

To the best of our knowledge we have demonstrated for the first time that β-lactoglobulin has the ability to stimulate the secretion of NO from immune cells. Others have found NO stimulatory activity of various milk proteins in different cell lines. Ringseis et al. (2005) demonstrated casein hydrolysate stimulates NO secretion from endothelial cells. Hayashida et al. (2004) found that NO was involved with bovine lactoferrin induced antinociception, however, a direct stimulatory effect between lactoferrin and NO was not established. Hayashida et al. (2004b) also demonstrated that hypotensive activity on endothelial cells of bovine lactoferrin was due to NO stimulation. Furthermore, lactoferrin had antifungal properties partially through stimulating production of NO by macrophages (Wakabayashi et al., 2003). However, we found that lactoferrin was unable to stimulate NO oxide secretion from naïve macrophage cells.

As our results demonstrate, the therapeutic potential of applying heat killed bacteria or milk derived protein in the modulation of disease, particularly cancer, through the manipulation of NO is present and merits further research. Sarcoma (Xie et al., 1995a), melanoma (Xie et al., 1995b), mouse fibrosarcoma (Cui et al., 1994) and mastocytoma cells (Kitajima et al., 1994) all undergo apoptosis when exposed to NO. Indeed our experimental results demonstrate increased apoptotic activity in HT29 human colon cancer cells when exposed to these strains of bacteria and milk derived proteins. Perhaps, these treatments are able to stimulate generation of potent apoptotic inducer NO from HT29. In addition, epithelial cells and macrophages within the body are in close communication, perhaps co-stimulation of both of these cells by our bacterial and milk protein treatments could induce apoptotic cellular death via NO stimulation.

In vivo, the tumourigenesis and metastatic potential of melanoma cells were dramatically repressed, when the melanoma cells were stimulated to express iNOS (Dong et al., 1994); delivery of iNOS expressing cells into colon and ovarian cancer resulted in 54% and 100% killing activity, respectively (Xu et al., 2001). Moreover, NO has been shown to inhibit angiogenesis, a key indicator of malignancy, via increases in iNOS expression, activity and ultimately NO production (Pipili-Synetos, et al., 2000) and upregulation of potent NO inducer, IL-2 (Sakkoula et al., 1997). Indeed, researchers have found that the addition of NO releasing moieties to standard nonsteroidal anti-inflammatory drugs, enhanced anti-inflammatory activity (Davies et al., 1997; Wallace et al., 1999) and accelerated healing in gastric ulcers (Elliot et al., 1995); in rats, inhibition of iNOS by iNOS inhibitor aminoguanidine resulted in increased colonic epithelial TNBS induced colitis, suggesting a protective role for NO (Dikopoulos, 2001).

Our results show that LAB and milk derived proteins, can significantly stimulate NO production from mouse macrophages. This represents a possibly potent, yet well tolerated and economic source of dietary immuno-agents in the therapeutic modulation of cancer.

4.3.2 The Effect of Dairy Derived Milk Proteins and Bacteria on *Salmonella typhimurium* LPS stimulated RAW 264.7 Mouse Macrophages

We examined the effects of these treatments on NO production in macrophage cells stimulated with *Salmonella typhimurium* LPS (Table 4.3). In the stimulated cells, our results were quite interesting. For all of the bacterial strains, the live, fermented supernatants and the ADA 03 peptidoglycan significantly ($p \leq 0.05$) inhibited the production of NO back to levels similar to the negative controls. However, the effect of heat killed cells were not as potent. Several of the heat killed cells (Table 4.3) demonstrated inhibitory activity, however not 100% inhibitory activity, decreasing NO production between 8.72 - 43.1% ($p \leq 0.05$) that of the control. Interestingly, several of the heat killed bacteria augmented the production of NO, the most dramatic augmentation came from NRC 13019 and YC 085, where augmentation was 34.1-40.6% above that of the control, however, this was not statistically significant.

These findings are quite interesting because, these bacterial strains were able to counteract the powerful immunostimulatory effects of LPS, this has important possible implications in the treatment of disease such as inflammatory bowel disease, where hyperproduction of NO contributes to or exacerbates the inflammatory condition (Kolios, et al., 2004; Head and Jurenka, 2003). Increased iNOS expression is associated with colonic inflammation in epithelial cells (Kolios et al., 1998), via cytotoxic action or activation of neutrophils (Ribbons et al., 1995), vasodilation (Middleton et al., 1993), nitrosamine production (Ohshima et al., 1994; Liu et al., 1995), the formation of the highly cytotoxic peroxynitrite radical (Wink et al., 1996; Kolios et al., 1995) and upregulation of proinflammatory mediator COX-2 (Resta-Lenert and Barrett, 2002).

Indeed treatment of colitis with probiotics in animal models shows positive results. The administration of *Lactobacillus* species in interleukin 10 knockout mice decreased deleterious pathogenic organism colonization and prevented colitis (Madsen et al., 1999). In mice genetically modified to lack the functional gene for iNOS (iNOS$^{-/}$), TNBS challenge did not induce inflammation, whereas in normal wild-type mice producing NO there was bloody diarrhea, mucosal edema or hemorrhage and increased nitrosative damage to tissue; supporting the hypothesis that NO is toxic and proinflammatory in IBD (Zingarelli et al., 1999).

Indeed, inhibition of NO synthase results in downgrading or attenuation of experimental colitis (Rachmilewiz et la., 1995c; Kankuri et al., 2001). Cystic fibrosis (CF) and IBD are associated with intestinal inflammation and increased rectal production of NO (Canani et al., 2002). In clinical trials, oral administration of *Lactobacillus GG* to children with CF/IBD resulted in a significant decrease in inflammatory marker NO and subsequently in inflammation (Bruzzese et al., 2004). In adults, VSL#3 was used successfully to maintain remission in ulcerative colitis patients, who were allergic to conventional treatment (Venturi et al., 1999); *E.coli* strain Nissle 1917, was found to be equally as effective as conventional treatments (mesalazine) at maintaining remission in ulcerative colitis patients (Rembacken, 1999). Further more, a strong correlation between NO and IBD severity has been established (Goggins et al., 2001; Vento et al., 2001; Koek et al., 2002; Guihot et al., 2000; Kimura et al., 1992). In fact, Rachmilewitz and colleagues measured mucosal NO production using NO electrodes in rectums of patients with ulcerative colitis confirmed increased iNOS activity and NO production. They also found that this correlated well with the clinical and endoscopic indices of disease activity (1998). Using animal models, Grisham and colleagues found the administration of an inhibitor of NO was able to decrease inflammatory scores within rats (1994).

Yet others have found limited effects of probiotics in the attenuation of IBD. In mice, the administration of *L. plantarum* species 299, was not able to improve intestinal permeability or downgrade disease progression (Kennedy et al., 2000); the administration of *Lactobacillus GG* in patients with Crohn's disease, failed to induce or maintain patients in remission (Schultz et al., 2004). In patients with Crohn's and colitis, NO and iNOS activities are significantly augmented compared to normal patients (Rachmilewitz et al., 1995; Kimura et al., 1998; Herulf et al., 1998).

Our experimental treatments were not uniform in their NO suppressive action. Several of the heat killed treatments did not completely inhibit secretion of NO like that observed with live and fermented milk treatments; perhaps physiologically these inhibitions are more realistic and relevant, since NO is not only injurious, but a fundamental component in many protective physiological processes, such as gut mucosal protection (Kolios et al., 2004). However, at high levels of inhibition such as observed in the live cells and the supernatants, the significant decrease in NO produced by iNOS may help downgrade serious or chronic inflammation that is involved in the pathology of IBD. Thus selective application of bacteria with regard to therapeutic outcome would be warranted.

However, caution should be exercised when drawing conclusion from these studies, in particular, the use of these animal models, does not accurately represent conditions in IBD. These models represent acute mucosal injury. New models, representing gradual or chronic inflammation leading to colitis from the administration smaller and more prolonged, continuous application of inflammatory compounds represents more physiologically relevant conditions of chronic inflammation observed in human IBD (Kolios et al., 2004). Researchers have shown, highly selective inhibitors of iNOS, reduced colonic NO activity and attenuated disease conditions in such models (Obermeier et al., 1999b; Krielstein et al., 2001), whereas same conditions aggravated acute colitis induced by single dose inflammatory conditions (Garvey et al., 1997). This suggests that the focus of research should perhaps focus on selective modification of iNOS activity and perhaps, in the relative reduction of NO levels as opposed to complete abrogation. In disease states where the level of NO inhibition needs to be higher, choosing iNOS inhibitors according to activity would be more appropriate.

Thus our results demonstrate a potential therapeutic role of dairy derived LAB/milk proteins as potent or moderate mediators of NO production in immune cells. However, further study is required to further elucidate mechanisms of action and physiological dosage require to elicit an appropriate response in humans.

4.4. CONCLUSIONS

The present experiment demonstrates the ability of various dairy derived LAB and milk proteins to affect macrophage production of NO. NO is a potent modulator of immune response both in colon cancer and inflammatory bowel disease. We have shown that various species/components of LAB and milk proteins can possibly be used as adjuvants in the modulation of these diseases; however, it should be cautioned that the immunological picture is not as simplistic. A large problem with using immunomodulating substances in the treatment of cancer and inflammation is that many of the cytokines and cell signaling mediators play paradoxical roles in disease progression and resolution; depending upon factors such as concentration of the mediator, stage of the disease, location of the disease and many other complex factors NO can have a beneficial or deleterious effect. Thus before dairy derived LAB and milk proteins can be ascribed as chemotherapeutic or IBD dietary adjuvants to treatment, further extensive research needs to be performed.

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Figure 4.1 Nitric Oxide Standard Curve (Griess Reaction)

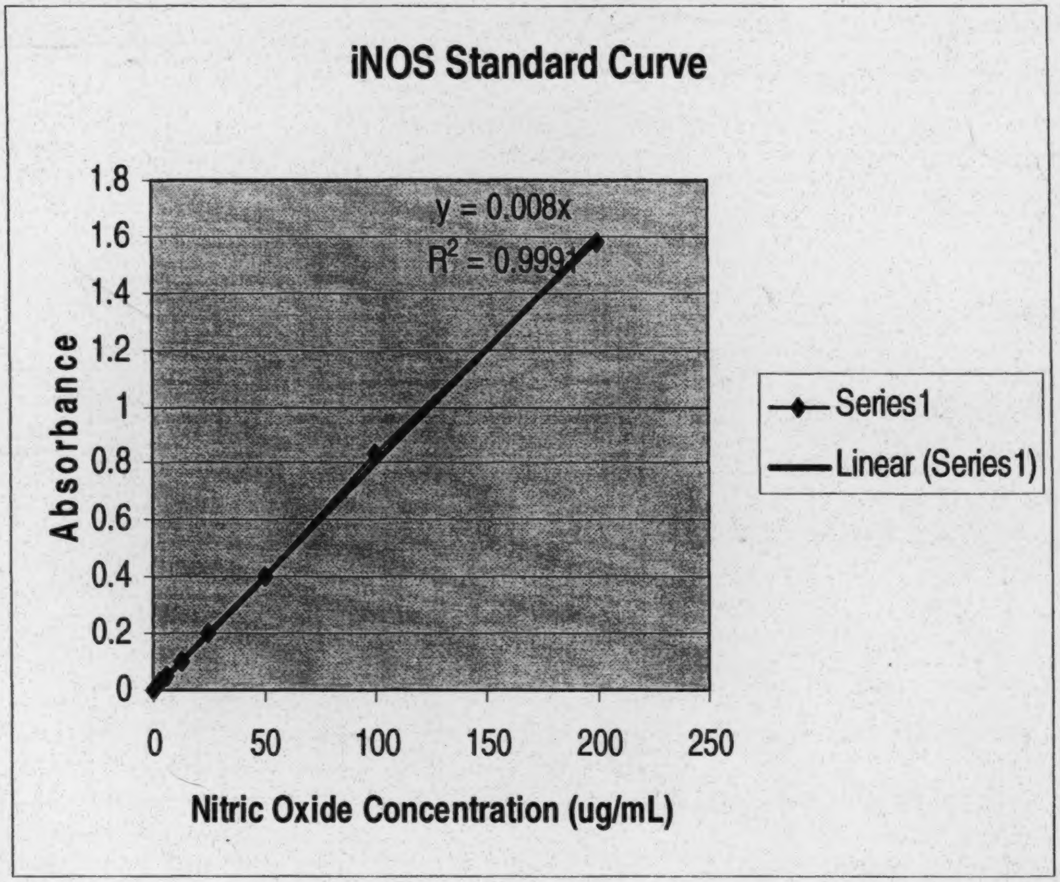

Table 4.1. Induction of nitric oxide production by dairy Lactic Acid Bacteria in unstimulated RAW 264.7 mouse macrophage cell line[a].

Lactic Acid Bacteria	Nitric Oxide (ug/mL)			
	Treatment			
	Heat Killed	Peptidoglycan		
		1000 μg/mL	800 μg/mL	600 μg/mL
L. acidophilus NRC 13019	19.54±2.05*	–	–	–
L. acidophilus NRC 13017	3.47±0.04*	–	–	–
L. casei ADA 03	6.59±0.09*	10.77±0.3*	14.94±0.4*	15.21±0.32*
L. casei NRC 13005	12.48±0.9*	–	–	–
L. acidophilus MR 100	17.75±0.5*	–	–	–
L. bulgaricus MR 110	5.77±0.6*	–	–	–
L. casei CSCC 2601	5.90±0.14*	–	–	–
Yogurt Culture YC 085	27.15±0.86*	–	–	–
Mixture	10.38±0.39*			

* Significantly different than the negative control ($p \leq 0.05$);-: Not tested; Positive Control: 31.89±0.30

a. Unstimulated RAW 264.7 cells (1×10^6 cells/well) were incubated with the previously described treatments for 48 hours at 37°C (5% CO_2/95% air). After incubation, the plates were centrifuged at 3000 rpm for 10 minutes and the supernatants were collected. Nitric oxide was measured using the Griess reaction. Immediately prior to the assay equal amounts of N-(1-naphthyl)ethylenediamine dihydrochloride (100 mg dissolved in 100 mL Milli Q water) and sulfanilamide (1 g/100 mL of 5% phosphoric acid) were combined together to create the Griess reagent. In optically clear 96 well flat bottom plates, 100 μL of treatment supernatant and 100 μL of Griess reagent were added to each well. The plates were incubated at room temperature for 30 minutes to allow development and stabilization of the chromophore. Absorbance was measured at 550 nm using a microplate reader.

Table 4.2. Induction of nitric oxide production by dairy derived casein and whey protein in unstimulated RAW 264.7 mouse macrophage cell line[a].

Milk Protein	Concentration	Nitric Oxide (ug/mL)
Casein Hydrolysate	40 mg/mL	2.24±1.65*
	20mg/mL	7.70±1.34*
	10 mg/mL	4.96±1.47*
Sodium Caseinate	75 mg/mL	8.80±1.28*
	37.5 mg/mL	2.99±0.37*
	18.75 mg/mL	3.91±2.19*
β-Lactoglobulin	120 mg/mL	1.30±0.04*
	90 mg/mL	1.76±0.04*
	60 mg/mL	8.75±0.70*
	30 mg/mL	8.45±0.99*
	15 mg/mL	1.54±0.11*

* Significantly different than the negative control ($p \leq 0.05$);-: Not tested

a. Unstimulated RAW 264.7 cells (1×10^6 cells/well) were incubated with the previously described treatments for 48 hours at 37°C (5% CO_2/95% air). After incubation, the plates were centrifuged at 3000 rpm for 10 minutes and the supernatants were collected. Nitric oxide was measured using the Griess reaction. Immediately prior to the assay equal amounts of N-(1-naphthyl)ethylenediamine dihydrochloride (100 mg dissolved in 100 mL Milli Q water) and sulfanilamide (1 g/100 mL of 5% phosphoric acid) were combined together to create the Griess reagent. In optically clear 96 well flat bottom plates, 100 µL of treatment supernatant and 100 µL of Griess reagent were added to each well. The plates were incubated at room temperature for 30 minutes to allow development and stabilization of the chromophore. Absorbance was measured at 550 nm using a microplate reader.

Table 4.3. Nitric Oxide secretion by *Salmonella typhimurium* LPS stimulated RAW 264.7 mouse macrophage cells treated with dairy derived probiotics

Probiotic	Treatment	Nitric Oxide		
		ug/mL	**Percent Inhibition[a]**	**Percent Augmentation[b]**
***L. acidophilus* NRC 13019**	Heat Killed	34.91±.1.17[¥]	-	34
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
***L.acidophilus* NRC 13017**	Heat Killed	14.82±0.44*	43.1	-
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
***L. casei* ADA 03**	Heat Killed	18.84±0.31*	27.6	-
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
***L. casei* NRC 13005**	Heat Killed	26.02±0.97*	-	5.3
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
***L. acidophilus* MR 100**	Heat Killed	23.77±0.92*	8.72	-
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
***L.bulgaricus* MR 110**	Heat Killed	29.68±1.3		11.1
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
***L. casei* CSCC 2601**	Heat Killed	18.64±0.87	28.42	-
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
Yogurt Culture YC 085	Heat Killed	36.61±0.45[¥]		40.6
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
Mixture	Heat Killed	0	100	-
	Live	0	100	-
	24 SN	0	100	-
	48 SN	0	100	-
LPS Control	-	***26.04***	-	-

RAW 264.7 cells (1×10^{6} cells/well) stimulated with *Salmonella typhimurium* LPS (1ug/mL) were incubated with the previously described treatments for 48 hours at 37°C (5% CO_2/95% air). After incubation, the plates were centrifuged at 3000 rpm for 10 minutes and the supernatants were collected. Nitric oxide was measured using the Griess reaction. Immediately prior to the assay equal amounts of N-(1-naphthyl)ethylenediamine dihydrochloride (100 mg dissolved in 100 mL Milli Q water) and sulfanilamide (1 g/100 mL of 5% phosphoric acid) were combined together to create the Griess reagent. In optically clear 96 well flat bottom plates, 100 µL of treatment supernatant and 100 µL of Griess reagent were added to each well. The plates were incubated at room temperature for 30 minutes to allow development and stabilization of the chromophore. Absorbance was measured at 550 nm using a microplate reader.

a % Inhibition and b: % Augmentation: represents percentage change in nitric oxide secretion as compared to control ; * Inhibition: Significantly different than the positive control ($p \leq 0.05$); [¥]Augmentation: significantly different than the positive control ($p \leq 0.05$)

Table 4.4. Nitric Oxide secretion by *Salmonella typhimurium* LPS stimulated RAW 264.7 mouse macrophage cells treated with dairy derived whey and casein proteins

		Nitric Oxide		
Protein	**Treatment**	**ug/mL**	**% Inhibition[a]**	**% Augmentation[b]**
Casein Hydrolysate	**40 mg/mL**	0	100*	-
	20 mg/mL	15.93±1.47	38.82*	-
	10 mg/mL	24.65±0.68	3.38*	-
Sodium Caseinate	**75 mg/mL**	28.46±2.0	-	5.99
	37.5 mg/mL	20.97±0.88	10.75*	-
	18.75 mg/mL	20.84±0.58	19.97*	-
β-lactoglobulin	**120 mg/mL**	0.16±0.39	99.03*	-
	90 mg/mL	0.37±0.24	98.23*	-
	60 mg/mL	4.12±0.51	84.02*	-
	30 mg/mL	8.05±0.58	69.09*	-
	15 mg/mL	28.93±0.96	-	1.88
β-casomorphin-7	**125 μg/mL**	28.61±0.69	-	9.87
	62.5 μg/mL	27.47±0.90	-	6.34
	31.25 μg/mL	28.11±0.61	-	7.95
Lactoferrin	**500 μg/mL**	24.98±0.75	14.2*	-
	200 μg/mL	25.58±1.59	1.77*	-
	100 ug/mL	25.07±1.49	3.73*	-
	20 μg/mL	21.41±0.18	17.76*	-
α lactalbumin	**35 μg/mL**	29.79±1.14	-	14.40
	25 μg/mL	27.95±1.51	-	7.03
	10 μg/mL	28.00±0.85	-	7.53
LPS Control	**-**	***26.04***	**-**	**-**

RAW 264.7 cells (1×10^6 cells/well) stimulated with *Salmonella typhimurium* LPS (1ug/mL) were incubated with the previously described treatments for 48 hours at 37°C (5% CO_2/95% air). After incubation, the plates were centrifuged at 3000 rpm for 10 minutes and the supernatants were collected. Nitric oxide was measured using the Griess reaction. Immediately prior to the assay equal amounts of N-(1-naphthyl)ethylenediamine dihydrochloride (100 mg dissolved in 100 mL Milli Q water) and sulfanilamide (1 g/100 mL of 5% phosphoric acid) were combined together to create the Griess reagent. In optically clear 96 well flat bottom plates, 100 μL of treatment supernatant and 100 μL of Griess reagent were added to each well. The plates were incubated at room temperature for 30 minutes to allow development and stabilization of the chromophore. Absorbance was measured at 550 nm using a microplate reader.

a. % Inhibition b. % Augmentation: represents percentage change in nitric oxide secretion as compared to control; * Significantly different than the positive control ($p \leq 0.05$)

CHAPTER 5[*]

Cytotoxicity of Probiotics and Milk Derived Bioactive Proteins/Peptides in HT29 Human Colon Cancer Cell Line

5.1 INTRODUCTION

The perception of food has changed dramatically throughout the history of man, particularly today with the emergence of the concept of functionality of foods. Functional foods refers to how bioactive substances found naturally in food may modulate our risk for disease through its effects on physiologic or other functional processes occurring within the body. An emerging area of interest within the realm of functional foods is that of probiotics, living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Fuller 1986). Lactic acid bacteria (LAB) consumption, particularly the *lactobacillus* sp., has been associated with a variety of health benefits including enhanced immune performance (Yatsutake et al., 1999), increased resistance to infectious diseases (Savadogo et al., 2004)) and suppression of cancer development (Aso et al., 1995). In addition, increasing evidence indicates that biotransformation of bacterial medium during growth or fermentation produces bioactive and potentially anticancer compounds (Gobbetti et al., 2002; Ruas-Madiedo et al., 2002).

Cancer is a neoplastic disease in which rates of occurrence have increased dramatically in the last few decades (World Cancer Research Fund, 1997). Many factors are thought to contribute to the formation of cancer, including an altered microflora and chronic inflammation. However, recently evidence is mounting that diet and food derived bioactive compounds can modulate the risk of cancer development (Vecchia and Negri, 1996; Wollowski et al., 2001). In particular, dairy derived LAB and milk protein/peptides have been found to have antitumour activity in a variety of *in vitro* and

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in vivo studies (Arimochi et al., 1997; Aso et al., 1995; Eliassen et al., 2002). Antitumour action of LAB and bioactive proteins/peptides have generally been attributed to their ability to modulate carcinogen action (Tavan et al., 2002), antimutagenic activity (Park and Rhee, 2001), alteration in indigenous bacterial enzyme expression (Ouwehand et al., 2002) or immunostimulation (Matsuguchi et al., 2003). However, there is evidence that LAB and bioactive proteins/peptides have antitumour activity through cytotoxic mechanisms (Fichera and Giese, 1994; Seow et al., 2002).

It is well known that there are two distinguishable mechanisms of cellular death; necrosis and apoptosis. Necrosis is classified as "accidental death" that often occurs when the cell is exposed to some physical or chemical treatment while apoptosis, is a natural cell death process by which old or unusable cells are removed. Cytotoxicity does not define a specific cellular death mechanism. It is simply the cell killing property of a chemical compound (such as that found in a food, cosmetic or pharmaceutical) or a mediator cell (such as that found in a cytotoxic T cell), independent from the mechanisms of death (Roche Applied Science, Apoptosis, Cell Death and Cell Proliferation Manual). The purpose of the present study was to determine whether dairy derived LAB and proteins/peptides have cytotoxic activity against HT29 Human colon cancer cells.

5.2 MATERIALS AND METHODS

5.2.1 HT29 Human Colon Cancer Cell Line Propagation and Growth

HT29 human colon cancer cell line (ATCC HTB-38) was kindly provided by Joan Turchinsky (Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, Alberta). HT29 cells were grown following the procedure recommended by ATCC. Briefly, an aliquot of cell culture HT29 from frozen stock cultures (-80°C) was grown in 75 cm^2 vented tissue culture flasks (Corning, Fisher Scientific, Nepean, Ontario, Canada) with McCoys 5A Modified Medium (with 1.5 mM L-glutamine/0.026mM sodium bicarbonate; Sigma Aldrich, St. Louis, MO, U.S.A) supplemented with 10% fetal calf serum(FCS; Gibco Laboratories, Chagrin Falls, IL, U.S.A.).

HT29 cells were routinely grown at 37°C with 5% CO_2/95% air atmosphere incubators. Spent cell culture medium was replaced every 2 to 4 days, depending upon cell culture density. Cells were routinely passaged with 0.25% trypsin – 0.53 mM EDTA solution (Sigma Aldrich) when monolayers reached 80% confluence. Cell number and viability were assessed using trypan blue exclusion with a haemocytometer. Aliquots (1mL) of HT29 stock cultures suspended in FCS (Gibco) supplemented with 10% tissue culture grade DMSO (Sigma Aldrich) was stored at -80°C until required for experimental use or for routine subculturing.

5.2.2 Probiotic LAB Propagation and Growth

Eight strains of LAB, *Lactobacillus acidophilus* MR 100, *Lactobacillus bulgaricus* MR 110, *Lactobacillus acidophilus* NRC 13017, *L.acidophilus* NRC 13019, *Lactobacillus casei* ADA 03, *Lactobacillus casei* NRC 13005, *Lactobacillus casei* subsp. *Casei* CSCC 2601 and Yogurt Culture YC 085 were used in this experiment. Stock of each bacterial strain was stored at -70°C in MRS broth containing 20% glycerol (Sigma Aldrich).

For experimental testing, these strains were grown in MRS broth (Difco, Detroit, MI, USA) at 37°C for 24 hours (bacterial cell density 1×10^9 cells/mL). The cells were washed three times with PBS. After each wash, cell suspension was centrifuged at 10,000 g for 2 minutes to collect cells. The bacterial cells were then resuspended in McCoys Modified 5A medium (v/v 10% FCS) without antibiotic/antimycotic immediately prior to cytotoxicity testing.

Portion of each strain of LAB (see above) was heat killed. The cells were washed three times in PBS. The cells were then placed into a 60°C water bath and incubated for 60 minutes. The heat killed cells were then centrifuged (10, 000 g for 2 minutes) and resuspended into McCoys Modified 5A medium. The heat killed cells were stored at -20°C until used.

5.2.3 *L. casei* ADA 03 Peptidoglycan Isolation and Purification

Peptidoglycan was isolated from *L.casei* ADA according to methods reported by De Ambrosini et al. (1996). Briefly, resuspended cells of *L.casei* in PBS were subjected to mechanical disruption using a French Press. Broken cells were centrifuged (10 000 g) for 10 minutes and the pellet obtained was suspended in 4% boiling sodium dodecyl sulfate (SDS; Sigma Aldrich) in order to dissolve the cell wall components. After 18 hours of incubation, the suspension was centrifuged at 20,000 g for 20 minutes, and washed with PBS to remove SDS. The pellet was then treated with RNase and DNase to remove nucleic acids, followed by washing with PBS. The pellet suspended in PBS was then treated with trypsin to digest any cell wall associated proteins. This preparation of cells was then washed with PBS and treated with 2% SDS for 4 hours to remove all proteins. Then the sample was washed thoroughly with PBS and deionized water (Milli Q, Millipore Corporation, Concord, California, U.S.A.). This fraction was considered as the whole cell wall preparation.

The insoluble cell wall precipitate was then treated with 10% trichloroacetic acid (Sigma Aldrich) for overnight period to remove teichoic acid from the cell wall. The suspension was centrifuged and the pellet was washed thoroughly with PBS and Milli Q deionized water. This pellet was taken as peptidoglycan and used for cytotoxicity testing at final concentrations of 1000, 800 and 600 μg/mL suspended in McCoys Modified 5A media.

5.2.4 Isolation of Fermented Milk Supernatant

A 1% (v/v) inoculum of the previously mentioned eight LAB was inoculated into 12% (w/v) skim milk containing 1% glucose and 1% yeast and incubated for 24 or 48 hours. The fermented milk samples were then centrifuged at 10, 000 g for 2 minutes. The supernatant collected was filtered with a syringe filter (0.25 μm pore size; Acrodisc, Gelman Sciences Ann Arbor, MI, U.S.A.). Aliquots of the supernatants were stored at -20 °C until required for cytotoxicity testing.

5.2.5 Milk Derived Bioactive Peptide Preparation

Sodium caseinate, β-lactoglobulin, lactoferrin, α-lactalbumin, casein hydrolysate and β-casomorphin-7 were all purchased from Sigma Aldrich (St. Louis, MO, USA). The following concentrations were used for each protein/peptide fraction: sodium caseinate: 75, 37.5 and 18.75 mg/mL; β-lactoglobulin: 120, 90, 60, 30, and 15 mg/mL;

lactoferrin (LF): 500, 200, 100, 20 ug/mL; α-lactalbumin: 35, 25 and 10 ug/mL; casein hydrolysate: 40, 20 and 10 mg/mL and β-casomorphin-7: 125, 62.5 and 31.25 ug/mL. Each protein/peptide solution was made in McCoys Modified 5A medium supplemented with 10% FCS and filtered with a 0.25 μm syringe filter (Acrodisc, Gelman Sciences). The sterile solutions were made immediately prior to cytotoxicity testing.

5.2.5 Cytotoxicity Assay

The cytotoxic action of milk and probiotic derived bioactive components were tested with the Cytotoxicity Detection Kit (Lactate Dehydrogenase: LDH) from Roche Diagnostics (Laval, Quebec, Canada). The tests were performed according to methodology outlined in the instruction manual provided by the supplier.

5.2.6 Statistical Analysis

Statistical Analysis was carried out in the Statistica program (Statistica 6.0). The data was analyzed through analysis of variance, Tukey's test, correlation of analysis and regression analysis.

5.3 RESULTS AND DISCUSSION

Increasing evidence indicates that food derived bioactive substances, particularly those from dairy associated products, can have antitumour potential (Eliassen et al., 2002; Ficher and Giese, 1994). Cancer is an extremely dynamic process, in which derailment in the balance between proliferation and cellular death contributes to tumour progression. Cancer prevalence, particularly colon cancer is increasing in North America (World Cancer Research Fund, 1997). Thus, the quest to isolate antitumour components and to determine mechanistic modes of action is heightening. The objective of this study was to determine whether dairy derived LAB and milk proteins/peptides have any direct cytotoxic action against a human colon cancer cell line (HT29).

5.3.1 The Cytotoxic Effect of Dairy Derived LAB and Milk Proteins/Peptides against HT29 Colon Cancer Cell Line.

The experiment was undertaken to evaluate the cytotoxic effects of these samples on HT29 human colon cancer cell line, through the measurement of lactic dehydrogenase (LDH). LDH is a stable cytoplasmic enzyme that is present in all cells; during cellular death, it is rapidly released into the cell culture supernatant. Samples studied in this experiment included: probiotic LAB (live and heat killed), peptidoglycan isolated from *L.casei* ADA 03, supernatants from 24 or 48 hour fermented milk and milk derived proteins/peptides (see above).

In general, we found that each treatment we tested had some degree of cytotoxic activity, however, the live treatments tended to have higher cytotoxic activity (Table 5.1). The strongest cytotoxic activity observed was from live cells of MR 100 and the 48 hour supernatant of NRC 13017, averaging 80.76%. Furthermore, these two samples were not significantly ($p>0.05$) different from the positive control, indicating potent cytotoxic activity. All of the live cells tested had cytotoxic activity ranging from 15.74% to 60.85%; while the heat killed cells had cytotoxic activity between 10.97-55.26%. In addition, the milk proteins we studied had cytotoxic activity (Table 5.2). Cytotoxic activity for milk proteins/peptides ranged from 0.96% (β-casomorphin-7: 31.25 μg/mL) to 41.25% ($p\leq0.05$; Lactoferrin: 100 μg/mL). Some protein concentrations tested did not exhibit any cytotoxic action.

In general, we found that live bacterial cells had stronger cytotoxic activity against HT29 colon cancer cells than the other treatments. Our results are in line with other researchers. Human isolates of *Enterococcus faecium* and *Lactobacillus salivarius*, has strong growth inhibitory effects on myeloma cancer cells (Zabala et al., 2001). *Lactobacillus plantarum* CBL/J isolated from ewes' milk cheese also had significant cytotoxic activity against myeloma cells; in addition, *L. plantarum* CBL/J also demonstrated significant inhibition of cytotoxicity induced by *N*-nitrosamines in Vero cells (African green monkey kidney cells) (Haza et al., 2004) . *L. sakei,* a LAB commonly used in meat preservations, was found to have the same cytotoxic qualities but did not have *N*-nitrosamine inhibitory qualities (Haza et al., 2005).

Furthermore, Kim and colleagues (2002) studied the cytotoxic effects of several strains of LAB (*L. acidophilus, L. bulgaricus, L. casei, L. plantarum, S. thermophilus, L. lactis* ssp. *lactis, L. lactis* ssp. *Cremoris, Bifidobacterium adolescentis, Bifidobacterium breve,* and *Bifidobacterium longum)* against colon, gastric, leukemia, lung, kidney, bladder, cervix, breast, hepatocarcinoma and prostate cancer cell lines. They found that several of the live strains and peptidoglycan had cytotoxic action against these cancer cell lines. Moreover, they also found that the cytoplasmic fraction isolated from these strains has profound cytotoxic activity, in some cases as high as 96% against these cell lines. This is a parameter that was not directly studied in the current experiment. However, we did observe that heat killed cells and peptidoglycan possessed cytotoxic activity, suggesting that some component present in the cell walls was partially responsible for the cytotoxic action exhibited by our dairy derived strains.

Furthermore, in *in vivo* studies LAB have demonstrated significant antitumour activity. A subcutaneous heat-killed mixture of *L. casei* strain Shirota (LC9018) was implanted along with bladder carcinoma cells in mice; after 21 days LC 9018 significantly suppressed tumour appearance and inhibited tumour growth as compared to the saline control (Takahashi et al., 2001). In rats fed freeze dried and live preparations of LAB there was a significant decrease in DMH induced large intestinal tumour burden and tumour mass and an absence of malignancy as compared to the control demonstrating significant antitumour activity (McIntosh et al., 1999; Goldin et al., 1996). Furthermore, subcutaneous injection of *L. casei* ADA 03 significantly inhibited the proliferation of Morris Hepatoma tumours implanted in rats (MacLeod et al., 1990).

In human clinical trials, *L. casei* strain Shirota decreased the recurrence of superficial bladder cancer after transurethral resection (Aso et al., 1992; Aso et al., 1995); furthermore, a negative correlation was observed with long term consumption of Yakult®, a fermented beverage containing *L. casei* strain Shirota, and the occurrence of bladder cancer (Ohashi et al., 2002). However, in clinical trials testing two probiotic preparations, Bioprofit ® (*L. rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* JS) and Rela ® (*L. reuteri* ING1), dismal results were observed for alteration in fecal bacterial enzyme azoreductase. Azoreductase is produced by deleterious members of the gastrointestinal microflora and has been implicated in the etiology of colon cancer. Although Bioprofit ® reduced azoreductase levels, the reduction in activity was small, thus the clinical significance of this decrease can not be determined (Ouwehand et al., 2002). Similar results were observed by Marteau et al., (1990).

Bacillus Calmette Guerin (BCG) is the gold standard treatment currently used for the treatment of bladder cancer. BCG acts via direct cytotoxic action against bladder cancer cells (Bohle, 2000). Interestingly, Seow et al. (2002) found that live/cell free supernatants of *L.casei* strain Shirota and *L. rhamnosus* strain GG, were just as cytotoxic to bladder cancer cells as standard treatment with BCG. Our experimental findings demonstrate similar results against colon cancer cells; however, cytotoxic action against bladder cancer cells requires further evaluation.

In the current experiment, we observed that cytotoxic action decreased at a certain concentration of peptidoglycan. The strongest cytotoxic action observed for peptidoglycan was at 600 ug/mL, whereas at concentrations greater than this cytotoxicity decreased (Table 5.1). This could be indicative of the number of receptor binding sites present on HT29 cells for peptidoglycan; at concentrations greater than 600 μg/mL these sites could become saturated. This is supported by the data from Fichera and Giese (1994), which showed that for *L. casei* ATCC 25180, 200 μg of peptidoglycan completely saturated all binding sites on Erlich ascites tumour cells. Correspondingly, maximum cytotoxic action was found at this concentration. Similar to our results Haza et al. (2004) also found a dose dependent cytotoxic response to *L. plantaram* CBL/J cells, supporting the hypothesis that cells have limited numbers of receptors for LAB binding. Indeed, Dziarski (1991) identified saturable peptidoglycan binding sites on mouse B and T lymphocytes and macrophages.

In addition, we found that cell free fermented milk supernatants had direct cytotoxic activity against HT 29 colon cancer cell lines; with NRC 13017 fermented milk exhibiting the highest degree of cytotoxic action (79.95%) among all the fermented milk supernatants (Table 5.1). This indicates that during fermentation through enzymatic or lactic acid digestion of the milk, some bioactive substances are released from their native protein structure (Sutas et a., 1996) or during growth, soluble bacterial metabolites with cytotoxic action are released (Ruas-Madiedo et al., 2002). Indeed, Manjunath and Ranganathan (1986) identified and isolated a cytotoxic substance from *L. casei* D-34 that had direct cytotoxic action against HeLa, HEp-2 and HFS-9 tumour cell lines. Ito et al. (1992) identified a cytotoxic compound produced through the fermentation of

Enterococcus faecium, and reported that the compound had significant growth inhibitory activity against cervical carcinoma cell line HeLa. Biffi et al. (1997) found that *Bifidobacterium infantis, Bifidobacterium bifidum, Bifidobacterium animalis, Lactobacillus acidophilus* and *Lactobacillus paracasei* fermented milk had significant cytotoxic activity against MCF breast cancer cells; they suggested that the presence of an ex novo soluble compound produced by LAB during fermentation is responsible for the pronounced cytotoxicity.

It has been suggested that biotransformation of growth medium, such as changes in pH, by-products of bacterial growth preparation, and/or bacterial toxins potentially mediate experimental outcome. Unfortunately these are not indicative or representative of *in vivo* conditions because these components are not always included in experimentation, most experimental studies use only purified cultures or components (Chen et al., 2005). This does not allow for the additive or synergistic effect of the biotransformation of growth medium to be expressed in experimentation. Indeed, in animal studies, Arimochi et al. (1997) found that *L. acidophilus* cell free supernatant possessed antitumour activity whereas the live cells did not. In the current study, we have attempted to circumvent this possible problem by using milk as the growth medium, thus all the bioactive ingredients liberated during the biotransformation of milk would also be retained and ingested.

Others have found similar results for biotransformation of growth medium by LAB. Park et al. (1998), found that fermentation of Korean mistletoe, a preparation used in traditional Chinese medicine to treat ailments such as hypertension and tumour, significantly increased its cytotoxic action against mouse tumour cell line MSV. Seven LAB (*Leuconostoc, Lactobacilli* and *Lactococci* strains) inoculated in Dolsan leaf mustard juice slightly increased cytotoxic activity against cancer cells as compared to uninoculated Dolsan leaf mustard juice (Yoo, et al., 2005); biotransformation of red ginseng extracts by LAB also increased cytotoxicity against mouse lymphocytic leukemia cell line (L1210), mouse lymphoid neoplasm cell line (P388) and human liver carcinoma cell line (HepG2) (Bae et al., 2004; Bae et al., 2003).

Using milk as the growth medium, Baricault et al. (1995), demonstrated that biotransformation of milk by *L. helveticus, Bifidobacterium, L. acidophilus* or a mix of *S. thermophilus* and *L. bulgaricus* had cytotoxic effects on HT29 colon cancer cells. In the present study we also studied bacterial biotransformed milk; we studied the supernatant of fermented milk, which represents the soluble whey protein fractions, as well as, several individual whey and casein proteins/peptides to determine which components in the fermented milk supernatant could be contributing to the cytotoxic effects of fermented milk. There are many bioactive peptides encrypted within protein sequences of milk. Proteolysis through bacterial enzymatic digestion, lactic acid degradation or food processing, releases peptides from the native structure enriching the milk (Gobbetti et al., 2002). Thus the biotransformation of milk through LAB fermentation may result in many possible cytotoxic substances that are released from the native milk protein structure.

We observed mild cytotoxic activity from several protein/peptide fractions (Table 5.2). Of the whey protein fractions, the highest degree of cytotoxic activity was observed with LF (100 μg/mL) it had 41.25% ($p \leq 0.05$) cytotoxic activity against HT 29 colon cancer cells, while the remaining whey protein treatments had only mild cytotoxic activity. Kanyshkova et al. (2003) found similar results for LF against L929 (mouse fibroblasts) and HL-60 (human promyelocytes). Conversely, others have found that LF has no cytotoxic activity, whereas lactoferrin derived peptide lactoferricin possessed significant cytotoxic activity against Meth A fibrosarcoma cells, melanoma cells and squamous cell carcinoma cell lines (Eliassen et al., 2002; Sakai et al., 2005). As observed from the conflicting results, perhaps cytotoxic potential varies according to cell line and concentration of LF used. However the results of this experiment and that of others demonstrate that perhaps whey proteins contribute to antitumour activity through direct cytotoxic action against cancer cells. Of the casein protein fractions, casein hydrolysate had the strongest inhibitory activity. Since casein hydrolysate is a mixture of peptides derived from different casein proteins, this would indicate involvement of synergistic action among peptides.

Conversely, Maeno et al. (2005) found that casein hydrolysate did not have any cytotoxic activity against Chinese hamster lung cells. However, the differences between our experimental outcomes and that of Maeno and colleagues (2005) could be due to the type of cells used. Maeno used non cancerous cells whereas our experiments were performed on cancer cell lines. As previously discussed oncogene/protooncogene activation state of a cell could alter therapeutic outcomes. In addition, the casein hydrolysate used by Maeno et al (2005) was produce via enzymatic digestion using *Aspergillus oryzae* protease; whereas our casein hydrolysate was produced via acid precipitation. Thus the bioactive peptide profile between the casein hydrolysate treatments could differ. Furthermore, the results of this experiment and that of Maeno et al (2005) demonstrate the cytotoxic activity of casein hydrolysate against cancerous cells and the sparing activity towards non cancerous cells.

Within milk, 20% of the total protein content is made up of whey (Marshall, 2004). The primary anticancer mechanism of action attributed to LF is through stimulation of glutathione, a free radical scavenger. Whey is rich in amino acid precursors required for glutathione synthesis. It is suggested, that whey protein may exert antitumour activity by providing the amino acids required for glutathione synthesis, stimulating the immune system, detoxification of carcinogens (Bounous, 2000) and iron binding capacity (Weinberg, 1996). Indeed in animal studies, whey proteins significantly decreased tumour incidence in 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary tumours in rats (Hakkak et al., 2000). In azoxymethane (AOM) induced colon carcinogenesis, administration of whey protein based diets, significantly lowered tumour incidence (Hakkak et al., 2001). Furthermore, in human clinical trials IMU Plus® (nondenatured whey protein) in co-administration with several nutraceutical products improved survival rates and quality of life parameters in end stage cancer patients; TNF α, glutathione and natural killer cells were all augmented by administration, suggesting that treatments as such are effective in late stage cancer treatment (See et al., 2002).

The present experiment demonstrates a possible mechanism of antitumour action for dairy derived LAB and milk proteins/peptides. Several of the treatments tested demonstrated direct cytotoxic action against colon cancer cells. Thus cytotoxicity may be another mechanism with which LAB and bioactive peptides/proteins derived from milk may exert antitumour action.

5.4 CONCLUSION

Several mechanisms have been attributed to LAB in regards to colon cancer inhibition. LAB have been found to bind and degrade potential carcinogens (Tavan et al., 2002), possess antimutagenic activity (Tavan et al., 2002), alter fecal bacterial enzyme activity (Ouwehand et al., 2002), play a role in immunomodulation (Matsuguchi et al., 2003) and mediate cytotoxic reactive nitrogen species (Hussain et al., 2003). In the present experiment, we have demonstrated the direct cytotoxic effect of various strains of dairy derived LAB and proteins/peptides against human colon cancer cell line HT 29. In addition, in other experiments performed in our laboratory we found that these treatments are able to stimulate tumour necrosis factor α, nitric oxide and inhibit interleukin 6 secretions from immune cells. These factors are potent anticancer immune parameters. Perhaps in combination with immune stimulatory activity, direct cytotoxic activity could also be a mechanism with which dairy derived LAB and milk proteins can exert antitumour activity. Furthermore, studies were performed in our laboratory to further elucidate if this direct cytotoxic activity is through apoptotic or necrotic mechanisms.

Despite the contradictory outcomes of clinical trials reported elsewhere (Ouwehand et al., 2002; Marteau et al., 1990) on the effect of probiotics on colon cancer, *in vitro* and *in vivo* animal studies show a role for LAB in the modulation of cancer (Takahashi et al., 2001; Kim et al., 2002). The current study demonstrates that several strains of LAB (live, heat killed and peptidoglycan), fermented milk supernatants and milk proteins/peptides have cytotoxic activity against colon cancer cell line HT 29. In order to obtain compounds with relevant activity *in vivo*, each compound needs to be well characterized. *In vitro* studies give us insight into the mechanistic perspective of how probiotics work. Perhaps in understanding *in vitro* action, we can exploit these positive attributes to develop *in vivo* preparations that are efficacious.

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Table 5.1 The Cytotoxic Effect of Dairy Derived LAB on HT29 Colon Cancer Cell Line[a]

Lactic Acid Bacteria	Treatment	% Cytotoxicity
L. acidophilus NRC 13019	Live	42.66±4.49*
	Heat Killed	55.26±3.50*
	24 Hour Supernatant	33.68±8.42*
	48 Hour Supernatant	59.13±16.75*
L. acidophilus NRC 13017	Live	60.85±16.75*
	Heat Killed	38.99±3.82*
	24 Hour Supernatant	14.79±1.49
	48 Hour Supernatant	79.95±16.72*†
L. casei ADA 03	Live	48.78±9.04*
	Heat Killed	14.54±1.92
	24 Hour Supernatant	73.56±19.59*
	PG 1000	6.99±0.14
	PG 800	8.02±1.05
	PG 600	12.22±5.10
L. casei NRC 13005	Live	54.92±4.80*
	24 Hour Supernatant	49.21±19.19*
	48 Hour Supernatant	59.78±5.77*
L. bulgaricus MR 100	Live	81.56±6.81*†
	Heat Killed	14.28±2.97
L. casei subsp. *casei* CSCC 2601	Live	15.74±0.18
	24 Hour Supernatant	31.79±2.33*
	48 Hour Supernatant	17.43±4.48
Yogurt Culture YC 085	Live	29.94±9.44*
	Heat Killed	10.97±0.36

[a] HT29 colon cancer cells were seeded into 6 well plates (6×10^6) and allowed to attach overnight (5% CO_2/95% air atmosphere incubator). Three milliliters of the previously described treatments were added to each well and incubated for 24 hours. The cell supernatants were aseptically collected centrifuged and filter sterilized to remove all residual treatment and cellular debris. The cytotoxicity of each treatment was determined by measuring the release of lactate dehydrogenase (LDH) from dying cells into the supernatant. LDH was measured using the Cytotoxicity Detection Kit (Roche Diagnostics, Laval, Quebec, Canada) Each experiment consisted of three assays. Each assay was performed in triplicate.

* Significantly different than the negative control ($p \leq 0.05$); †Not significantly different than the positive control ($p > 0.05$).

Table 5.2. The Cytotoxic Effect of Dairy Derived Milk Proteins/Peptides on HT29 Human Colon Cancer Cell Line[a]

Protein/Peptide	Concentration	% Cytotoxicity
Casein Hydrolysate	40 mg/mL	25.66±12.02*
Sodium Caseinate	75 mg/mL	17.71±6.85
	37.5 mg/mL	12.35±1.91
	18.75	11.27±3.26
β-Lactoglobulin	120 mg/mL	9.44±0.93
	60 mg/mL	19.11±6.14
	30 mg/mL	4.57±0.71
	15 mg/mL	16.41±2.73
β-Casomorphin-7	125 μg/mL	11.60±0.95
	62.5 μg/mL	18.26±0.62
	31.25 μg/mL	0.96±0.87
Lactoferrin	500 μg/mL	4.16±2.34
	100 μg/mL	41.25±6.88*
	20 μg/mL	7.91±1.17
α-Lactalbumin	25 μg/mL	6.02±0.55

[a] HT29 colon cancer cells were seeded into 6 well plates (6×10^6) and allowed to attach overnight (5% CO_2/95% air atmosphere incubator). Three milliliters of the previously described treatments were added to each well and incubated for 24 hours. The cell supernatants were aseptically collected centrifuged and filter sterilized to remove all residual treatment and cellular debris. The cytotoxicity of each treatment was determined by measuring the release of lactate dehydrogenase (LDH) from dying cells into the supernatant. LDH was measured using the Cytotoxicity Detection Kit (Roche Diagnostics, Laval, Quebec, Canada) Each experiment consisted of three assays. Each assay was performed in triplicate.

* Significantly different than the negative control ($p\leq0.05$);

CHAPTER 6*

Induction of Apoptosis and Necrosis by Dairy Derived Probiotics and Protein/Peptides against HT29 Human Colon Cancer Cell Line

6.1 INTRODUCTION

The perception of food has changed dramatically with the emergence of functionality of foods. Food is traditionally viewed as a source of nutrients for the body to grow and repair. The concept of functional foods has emerged to alter this concept. How food may affect or modulate our risk for disease through manipulation of physiological processes within the body is the key concept of functional foods.

Milk and dairy products represent a large class of functional foods. Milk is an extraordinary food that is naturally nutritious. Interest in the functionality of foods, has brought milk into the forefront of functional food research and development. Milk is not only an excellent source of vitamins, minerals and protein, but also milk protein contains within its native structure bioactive peptide sequences that are liberated through processing or fermentation.

Milk is composed of two major protein groups, casein and whey. Casein is a precursor for β-casomorphin-7, casokinins and caseinphosphopeptides. Whey is a precursor for many bioactive proteins/peptides, such as α-lactalbumin, β-lactoglobulin and lactoferrin (Schlimme and Meisel, 1995). Whey proteins have been associated with a variety of beneficial effects, including immunomodulation, antioxidation, antimicrobial and anticarcinogenesis (Korhonen et al., 1998); while there are fewer properties attributed to peptides of casein origin. Casein is involved in the regulation of a variety of processes such as analgesia, gastric emptying, antimutation, anticancer as well as immunomodulation (Korhonen et al., 1998; van Boekel, et al., 1993; Yoshida and Xiuyun, 1992; de Morena de LeBlanc and Perdigon, 2004).

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* Conference Proceedings: A portion of this research was presented at the European Conference on Probiotics and their Applications. Krakow, Poland. October 6th – October 8th, 2005. Poster Presentation: Induction of Programmed Cell Death by Probiotic Bacteria.

** Award: Most Innovative Research and Contribution to Research Area Award. European Conference on Probiotics and their Applications. Krakow, Poland, October 6th – December 3rd, 2005.*

* Conference Proceedings: A portion of this research was presented at the 10th World Congress of Clinical Nutrition. Phuket, Thailand. November 30th – December 3rd, 2004. Oral Presentation: The Role of Probiotics in Cytotoxicity and Apoptosis.

Through fermentation, bioactive peptides are liberated from their native protein structure. Fermentation of milk was done traditionally for preservation purposes and to develop desired organoleptic qualities, however, in the last few decades there has been emerging interest in the bacteria that are used to ferment milk. These lactic acid bacteria have been termed probiotics. Probiotics are defined as, living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Fuller, 1989).

Traditionally, lactic acid bacteria (LAB) were used in milk fermentation. LAB serves not only as a dairy ingredient that contributes to functional characteristics and flavor profiles, but also contributes to preservation by producing its primary metabolite, lactic acid. However, LAB and its associated growth products have been found to have beneficial effects on health, including tumour prevention, prevention of enteric infections, constipation (Salminen et al., 1998) and immunostimulation (Watanabe, 1996). A commonly used bacteria for yogurt fermentation, *Lactobacillus Rhamnosus* GG (LGG) has been found to be effective in the treatment of rotoviral infections (Szajewska et al., 2001), atopic dermatitis (Kalliomaki et al., 2001) and in the prevention and treatment of diarrhea (Marteau et al., 2001).

Cancer is a neoplastic disease in which rates of occurrence have increased dramatically. Many factors are thought to contribute to the formation of cancer, including diet, an altered microflora, chronic inflammation and environmental factors mechanism. Many studies suggest that dairy derived LAB and bioactive proteins, play a role in protection against cancer. However, there is little direct evidence that implicates specific effects on biological events of relevance to cancer development. The measurement of apoptosis provides a direct link between cancer and anticancer effects of probiotics and milk proteins. Apoptosis is natural cell death that is an innate cellular defense mechanism against cancer development. Augmentation or facilitation of apoptosis by probiotics and milk proteins increase the elimination of mutated cells that might otherwise progress to malignancy.

In colon cancer, there is a derailment of cellular death processes. Indeed, these diseases may often occur as a result of a continuous immune response from cells that have defective apoptotic mechanisms. For example, due to the chronic epithelial damage from a heightened or sustained immune response, inflammatory bowel disease can result in cancer in the colon (Kountouras et al., 2003).

In cells, there are two forms of cellular death, necrosis and apoptosis. Necrosis is death caused by cellular injury due to exposure to toxic chemicals or mechanical injury. Necrotic cell death is quite traumatic, involving cellular decomposition and leakage of cell contents, resulting in inflammation. Apoptosis, on the other hand, is natural cellular death that is often derailed in cancer. Apoptosis involves cell shrinkage, mitochondrial breakdown, and nucleus degradation and is eventually engulfed by phagocytic cells (i.e. macrophages and dendritic cells) to be resorbed back into the body (Kountouras et al., 2003). Regulation of cell death, that is a balance between cellular proliferation and cellular death, is imperative to normal homeostasis.

Many defective or dangerous cells are able to trick the apoptotic mechanisms within the body. Recent research has shown the possible use of probiotics and milk derived proteins to treat colon cancer (Di Marzio et al., 2001; Marchetti et al., 1997; Heerdt et al., 1997; Luhrs et al., 2002; Jan et al., 2002). In cancer research, there is much evidence indicating the role of apoptosis in cancer development and malignancy (Wynford-Thomas, 1996). Furthermore, treatment of bladder cancer via induction of necrosis has also been successfully used. This illustrates the need to search for potential bioactive agents that are able to induce cellular death via apoptotic or necrotic mechanisms of action. The purpose of this work is to assess the direct effect of several strains of dairy derived LAB and proteins on the apoptotic and necrotic activity against HT29 human colon cancer cells.

6.2 MATERIALS AND METHODS

6.2.1 HT29 Human Colon Cancer Cell Line Propagation and Growth

HT29 human colon cancer cell line (ATCC HTB-38) was kindly provided by Joan Turchinsky (Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, Alberta). HT29 cells were grown following the procedure recommended by ATCC. Briefly, an aliquot of cell culture HT29 from frozen stock cultures (-80°C) was grown in 75 cm^2 vented tissue culture flasks (Corning, Fisher Scientific, Nepean, Ontario, Canada) with McCoys 5A Modified medium (with 1.5 mM L-glutamine/2.2 g/L sodium bicarbonate; Sigma Aldrich) supplemented with 10% fetal calf serum (FCS; Gibco Laboratories, Chagrin Falls, IL, U.S.A.).

HT29 cells were routinely grown at 37°C with 5% CO_2/95% air atmosphere incubators. Spent cell culture media were replaced every 2 to 4 days, depending upon cell density. Cells were routinely passaged with 0.25% trypsin – 0.53 mM EDTA solution (Gibco) when monolayers reached 80% confluence. Cell number and viability were assessed using trypan blue exclusion with a haemocytometer. Aliquots (1mL) of HT29 stock cultures suspended in FCS (Gibco) supplemented with 10% tissue culture grade dimethylsulfoxide (DMSO; Sigma Aldrich) was stored at -80°C until required for experimental use or for routine subculturing.

6.2.2 Probiotic LAB Propagation and Growth

Eight strains of LAB, *Lactobacillus acidophilus* MR 100 (MR 100), *Lactobacillus bulgaricus* MR 110 (MR110), *Lactobacillus acidophilus* NRC 13017 (NRC 13017), *L.acidophilus* NRC 13019 (NRC 13019), *Lactobacillus casei* ADA 03 (ADA 03), *Lactobacillus casei* NRC 13005 (NRC 13005), *Lactobacillus casei* subsp. *Casei* CSCC 2601 (CSCC 2601) and Yogurt Culture YC 085 (YC 085) were used in this experiment. These strains were routinely grown in MRS broth (Difco, Detroit, MI, USA) at 37°C for 24 hours. Aliquots of stock bacterial strains were stored at -70°C in MRS broth containing 20% glycerol (Sigma Aldrich).

For cellular death experiments, these strains were grown in MRS broth (Difco, Detroit, MI, USA) at 37°C for 24 hours (bacterial cell density 1×10^9 cells/mL). The cells were washed three times with PBS. After each wash, cell suspension was centrifuged at 10, 000 g for 2 minutes to collect cells. The bacterial cells were then resuspended in McCoys Modified 5A medium (10% FCS) without antibiotic/antimycotic immediately prior to induction of programmed cell death.

The previously mentioned eight strains of LAB were heat killed. The cells were washed in PBS. After each wash, cell suspension was centrifuged at 10, 000 g for 2 minutes to collect cells. Washing was repeated three times. The cells were then placed into a 60°C water bath and incubated for 60 minutes. The heat killed cells were then centrifuged (10 000 g for 2 minutes), resuspended into McCoys Modified 5A medium. The heat killed cells were then stored at -20°C until used.

6.2.3 *L. casei* ADA 03 Peptidoglycan Isolation and Purification

Peptidoglycan was isolated from *L.casei* ADA 03 according to the methods reported by De Ambrosini et al., (1996). Briefly, resuspended cells of *L.casei* ADA 03 in PBS were subjected to mechanical disruption using a French Press. Broken cells were centrifuged (10 000 g) for 10 minutes and the pellet obtained was suspended in 4% boiling sodium docecyl sulfate (SDS; Sigma Aldrich) in order to dissolve the cell wall fraction. After 18 hours of incubation, the suspension was centrifuged at 20, 000 g for 20 minutes, and washed with PBS to remove SDS. The pellet was then treated with RNase and DNase to remove nucleic acids, followed by washing with PBS. The pellet suspended in PBS was then treated with trypsin to digest any cell wall associated proteins. This preparation of cells was then washed with PBS and treated with 2% SDS for 4 hours to remove all proteins. Then the sample was washed thoroughly with PBS and deionized water (Milli Q, Millipore Corporation, Concord, CA, U.S.A). This fraction was considered as the whole cell wall preparation.

The insoluble cell wall precipitate was then treated with 10% trichloroacetic acid (Sigma Aldrich) for overnight period to remove teichoic acid from within the cell wall. The suspension was centrifuged and the pellet was washed thoroughly with PBS and Milli Q water. This pellet was taken as peptidoglycan. For experimental testing, the pellet was suspended in McCoys Modified 5A media at concentrations of 1000, 800 and 600 µg/mL.

6.2.4 Isolation of Fermented Milk Supernatant

A 1% (v/v) inoculum of the previously mentioned LAB were inoculated into 12% (w/v) skim milk containing 1% (w/v) glucose and 1% (w/v) yeast and incubated for 24 or 48 hours. The fermented milk samples were centrifuged at 10 000 g for 2 minutes. The collected supernatant was filtered with a syringe filter (0.25 μm pore size; Acrodisc, Gelman Sciences, Ann Arbor, MI, U.S.A.). Aliquots of the supernatants were stored at -20 °C until required for induction of programmed cell death.

6.2.5 Milk Derived Bioactive Peptide Preparation

Sodium caseinate, β-lactoglobulin, lactoferrin, α-lactalbumin, casein hydrolysate and β-casomorphin-7 were all purchased from Sigma Aldrich (St. Louis, MO, USA). The following concentrations were used for each protein/peptide fraction: sodium caseinate: 75, 37.5 and 18.75 mg/mL; β-lactoglobulin: 120, 90, 60, 30, and 15 mg/mL; lactoferrin: 500, 200, 100, 20 μg/mL; α-lactalbumin: 35, 25 and 10 μg/mL; casein hydrolysate: 40, 20 and 10 mg/mL and β-casomorphin-7: 125, 62.5 and 31.25 μg/mL. Each protein/peptide solution was made in McCoy's medium supplemented with 10% FCS and filter sterilized with a 0.25 μm syringe filter disc (Acrodisc, Gelman Sciences). The sterile solutions were made immediately prior to experiments.

6.2.6 Apoptosis Assay (Cell Death Detection ^{PLUS})

The apoptotic activity against HT 29 colon cancer cells in the previously mentioned LAB and milk proteins was tested using the Cell Death Detection ELISA^{PLUS} Assay (Roche Diagnostics, Laval, Quebec, Canada). The experiment was performed according to supplier protocol.

6.2.7 Necrosis Assay (Cell Death Detection ^{PLUS})

The necrotic activity of eight strains of dairy isolated probiotic LAB and milk derived proteins against HT29 human colon cancer cells was tested using the Cell Death Detection ELISA^{plus} assay (Roche Diagnostics, Laval, Quebec, Canada). The experiment was performed according to supplier protocol.

6.2.8 Enrichment Factor Calculations

To measure apoptotic and necrotic activity, the enrichment factor specific to mono and oligonucleosomes released into the cytoplasm and spent culture media, respectively, was calculated using the following formula:

$$EF = \frac{mU \text{ of the sample (dying cells)}}{mU \text{ of negative control}}$$

- where mU represents the absorbance value.

6.2.9 Statistical Analysis

Statistical Analysis was carried out in the Statistica program (Statistica 6.0). The data was analyzed through analysis of variance, Tukey's test, correlation of analysis and regression analysis.

6.3 RESULTS AND DISCUSSION

6.3.1 Apoptotic and Necrotic Activity of LAB

Several strains of live probiotics tested had mild stimulatory effect on apoptotic activity (Table 6.2). In descending order of apoptotic activity, NRC 13005, NRC 13017, ADA 03, CSCC 2601, NRC 13019 and YC085. However, none of the treatments were statistically different than the control ($p \leq 0.05$).

Interestingly, several heat killed samples also had apoptotic activity (Table 6.2). However, apoptotic activity, with the exception of *L. bulgaricus* MR110 and *L. acidophilus* MR 100, was lower than the live samples. In descending order of apoptotic activity, NRC 13017, NRC 13005, ADA 03, CSCC 2601, MR 100, MR 110 and NRC 13019. It is interesting to note that live MR100 and MR 110 did not possess any apoptotic activity but the heat killed cells were able to activate some apoptotic pathway. We also tested peptidoglycan isolated from cell walls of ADA 03. All of the concentrations tested had slight but statistically insignificant apoptotic activity.

Furthermore, fermented milk supernatant was tested to determine whether apoptotic activity was attributed to the actual cell itself or some growth factor that is released into the growth medium during fermentation. All samples tested, with the exception of the 24 hour supernatant for *L. acidophilus* NRC 13019, did not possess any apoptotic activity. The 24 hour supernatant for *L. acidophilus* NRC 13019 demonstrated only slight, but statistically insignificant, apoptotic activity. However, these results do demonstrate that the eight strains of dairy derived LAB and milk fermented by these strains can induce mild apoptotic activity in HT29 colon cancer cells; furthermore, this suggests that part of the antitumour activity attributed to LAB could be due to apoptosis induction.

In cancer cells, there is a derailment of apoptosis, resulting in DNA damaged cells that escape cellular death mechanisms and ultimately result in malignancy. Jurkat cells (acute human T leukemia cell line) treated with sonicated *Lactobacillus brevis* (D2) and *Streptococcus thermophilus* (S244) had increased apoptotic activity, whereas, normal human peripheral blood lymphocytes treated with the same bacterium, did not have the same effect (Di Marzio et al., 2001). Indeed, Linsalata and colleagues (2005) tested the effect of a popular probiotic preparation, VSL#3, on bioamine synthesis and cellular proliferation parameters in normal cells. VSL#3 was able to significantly inhibit polyamine synthesis enzyme, ornithine decarboxylase (ODC), activity in the distal colon of rats and significantly augmented apoptotic activity. In SNU-1 human stomach adenocarcinoma cells, Kim et al. (2004), found that cytoplasmic fractions of *Lactococcus lactis* subsp. *lactis* had inhibitory activities on cellular proliferation. They associated this antiproliferative activity of *L. lactis* to the induction of apoptosis. Fichera and Giese (1995) attributed the apoptotic activity of *L. casei* ATCC25180 against several murine and human tumour cells lines to the bacterial cell wall preparation and peptidoglycan components of lactobacillus.

These studies suggest that the presence of live cells are not always required for bioactivity, heat killed cells are able to induce the activation of apoptotic pathways in cancer cells. Our results, demonstrate similar effect. Heat killed cells and peptidoglycan fractions were able to induce slight apoptotic activity in colon cancer cells. This has important implications. In the gastrointestinal tract, colon cells have direct contact with bacteria and bacterial components, thus these agents can directly induce apoptotic activity.

Although, many studies demonstrate the ability of probiotic bacteria to induce apoptosis, the mechanisms remain elusive and in many instances, contradictory. However, many postulates have been posed in regards to probiotic mechanisms of antitumour activity. Probiotics may have a differential effect on normal cells due to protooncogene or oncogene expression on normal versus malignant cells; or the ability of LAB to alter tumour cell surface components to generate cellular death messengers may also be a mechanism of antitumour activity (DiMarzio, 2001).

LAB is also thought to act via inhibition of polyamine synthesis. Polyamines are said to play an integral role in apoptosis. Polyamines are found in both normal and in neoplastic rapidly proliferating cells; the concentration is dramatically higher in neoplastic cells than in normal cells, where it is found in insignificant amounts. Polyamines synthesis relies on the conversion of arginine through a series of steps to ornithine, which is then decarboxylated by ornithine decarboxylase (ODC) (Linsalata et al., 1993; Linsalata et al., 2005). ODC is considered to be the first and rate-limiting step in polyamine synthesis. Probiotic bacteria are able to affect the course of polyamine synthesis in two ways. Firstly, some bacteria are able to produce an alternate enzyme, arginine deiminase, which competes for arginine, catalyzing the irreversible conversion of arginine to citruline and ammonia. The resultant decrease in arginine available within the cellular environment, contributes to a reduction of polyamine generation by rapidly proliferating cells. Transformation of cells through growth factor stimulation, carcinogens or viruses is characterized by elevated levels of polyamines, which is mediated by increased polyamine biosynthesis and/or enhanced uptake of polyamines (Pegg and McCann, 1982); a reduction in polyamine concentration has been associated with increased apoptotic activity (Thomas and Thomas, 2001). *L. brevis* was able to produce arginine deiminase (Di Marzio et al., 2001), thus the inhibition of polyamine formation may be the mechanism with which these probiotic bacterium exert apoptotic effect. Secondly, probiotic preparation, VSL#3, is able to decrease polyamine concentration within the intestinal environment through direct inhibition of ODC activity (Linsalata et al., 2005).

de Moreno de LeBlanc and Perdigon (2004) observed a significant increase in apoptotic cells in mice fed cyclically with yogurt (*L. delbrueckki* subsp. *bulgaricus* and *Streptococcus thermophilus*) after tumour induction by dimethylhydrazine (DMH). Rachid (2002) also observed increased levels of apoptotic bodies when yogurt was administered before and after tumour induction by DMH. They suggest that yogurt may affect the course of cancer development via apoptosis, during the progression or promotion stages of cancer. The elevated apoptotic response was attributed to up regulation of TNFα and IFNγ concentration, which favors apoptosis (de Moreno de LeBlanc and Perdigon, 2004; Perdigon et al., 2002; Sellers and Fisher,1999).

Since these studies administered yogurt as a whole product, it is not possible to determine whether apoptotic effect was due solely to the presence of probiotic bacterium in the fermented milk or if bioactive compounds are liberated during fermentation, which may have an effect on the course of cancer development. Propionibacteria, are commonly used in dairy production of cheeses such as Swiss cheese. During fermentation, propionibacteria convert carbohydrates and lactic acid into short chain fatty acids (SCFA). SCFA such as butyrate, propionate and acetate have been associated with increased apoptotic activity in colon cancer cells but not in normal cells (Jan et al., 2002; Marchetti et al., 1997, Le Leu et a., 2005, Le Leu et al., 2003). In addition, SCFA also induce differentiation and suppression of proliferation (Heerdt et al., 1997). Although, SCFA have mostly been found to be produced through fermentation by bacteria of the genera Propionibacterium. We wanted to determine if dairy isolated LAB produced a similar ex-novo compound that could induce apoptosis. In our study, we found that fermented milk supernatant from only one strain (NRC 019) had any apoptotic activity, suggesting that unlike Propionibacterium, the rest of the strains that we tested were not able to liberate any similar bioactive compounds. Further researcher needs to be done to determine and isolate the active compound liberated from milk by NRC 019.

Interestingly, we also found that not only do the strains of LAB tested induce apoptotic cell death, but is also involved in the induction of necrosis (Table 6.3). Several of the live and heat killed cells were able to induce necrotic activity in HT29 colon cancer cells. Live cells of, NRC 13019 and NRC 13017and heat killed YC085 all exhibited significant necrotic killing activity. Necrosis is known as accidental cell death. As opposed to apoptosis, necrosis is associated with cell lysis and consequent spilling of cytoplasmic contents, including enzymatic compounds into the extracellular fluid. *In vivo,* necrosis is associated with extensive tissue damage, ultimately resulting in the immune system mounting a massive inflammatory response. This form of cellular death is generally considered deleterious because it is normally caused by physical or chemical damage to the cell, and ultimately damage to adjacent cells. However, in the treatment of superficial bladder cancer, induction of necrosis is desirable and in fact facilitated by intravesical instillation of the probiotic *Mycobacterium bovis* (*Bacillus Calmette-Guerin (BCG)*). BCG treatment causes necrosis in bladder tumour cells, lysis of these cells results in the mounting of a large and injurious inflammatory response. It is this response that leads to the killing of neighbouring tumour cells, while at the same time facilitating removal of necrotic cells. It is widely accepted that BCG mediates tumour killing through this bystander effect (Bohle, 2000). However, others have found that BCG acts by inducing cell cycle arrest (Chen et al., 2005) or via immunomodulation (Zlotta, et al., 2000; Patard et al., 2003).

BCG immunotherapy is considered to be the gold standard in the treatment bladder cancer. It is highly effective at preventing the recurrence and progression of bladder cancer. However, it is not without side affects, including dysuria, a reduction in bladder capacity, urinary tract infections, fever, chills and malaise, as well as, an increased risk of systemic mycobacterium infection since the cells are live (Lockyer and Gillat, 2001; Mungan and Witjes, 1998; Paterson and Patel, 1998). Thus there is a need to search for alternative forms of treatment.

Biolactis powder, a powder formulation of *L.casei* strain *shirota,* suppressed recurrence of superficial bladder cancer after surgical transection. Heat Killed *L.casei* strain *shirota,* had more effective tumouricidal activity than BCG against murine bladder tumour (Aso et al., 1992; Aso et al., 1995). Seow et al. (2002) found that *L.casei* strain *Shirota* was just as effective as BCG at inducing bladder cancer tumour cell death. Indeed they found that *L.casei* strain *Shirota* induced death via necrosis. In our experiments, we found several live and heat killed strains possessed necrotic activity.

Live NRC 13019, NRC 017, ADA 03, NRC 13005, Y085 and all heat killed cells with the exception of NRC 13005 had necrotic activity. Statistically significant ($p \leq 0.05$) necrotic activity was observed in live cells of NRC 13019, NRC 13017 and in heat killed cells of YC 085. In addition, several of these also elicited apoptotic activity.

Since treatment of bladder cancer with BCG is associated with many side effects, the need for alternative treatments is needed. The LAB that we tested are all food grade, GRAS and of dairy origin, thus are well tolerated. Thus perhaps through the ability to induce necrotic activity, these strains could be potentially used as alternative or adjuvants to treatment. In addition, since these bacteria are not considered dangerous as live BCG are and exert an effect whether live or heat killed, handling of these organisms are much safer. The majority of cell death research done on LAB has focused on apoptosis; however, we have identified several strains of LAB that are necrotic. As discussed necrotic cell death is a treatment modality employed in the treatment of bladder cancer, further research is required to evaluate the potential of dairy derived LAB, in particular NRC 13019, NRC 13017 and YC085, in experimental models of bladder cancer.

6.3.2 Apoptotic Activity of Milk Derived Proteins

Although, much interest has been expressed for the use of milk as a functional food ingredient, little evidence exists for its role in driving the cell towards apoptosis. Milk contains within its native structure, many protein/peptide fractions that may have functional properties. Many of these bioactive peptides may be liberated from the native structure via enzymatic hydrolysis, proteolytic activity of LAB through microbial fermentation or gastrointestinal digestion (Sutas et al., 1996; Meisel and Bockelmann, 1999). Many biological activities have been attributed to proteins isolated from milk, including opioid activities (Hatzoglou et al., 1996a; Hatzoglou et al., 1996b), anticarcinogenic and immunoregulatory functions (Sutas et al., 1996; Eliassen et al., 2002) amongst many other biological activities (as reviewed by Shah, 2000). Whey and casein proteins have been shown to have inhibitory effect in *in vitro* studies (McIntosh et al., 1995) and *in vivo*, where both initiation and progression stages of cancer show inhibition (Bounous et al., 1988). In clinical trials, whey protein concentrate Immunocal

™, induced improvements in disease parameters in breast cancer patients (Kennedy et al., 1995).

6.3.2.1 Whey Protein Fractions

Lactoferrin, an 80 kDa glycoprotein, is an iron binding protein found not only in milk but in tears, saliva and seminal fluid. In humans, milk lactoferrin is found in higher concentrations than in cows' milk. Human colostrum and milk can contain 6-8 g/L and 2-4 g/L of lactoferrin, respectively while in cows' this value is 0.02-0.2g/L and 0.5 g/L, respectively. However, human and bovine lactoferrin have 69% identical amino acids, and their three dimensional structure is highly conserved (Baker et al., 2002; van Belzen, 2002). We tested between 0.02 to 0.5 g/L of bovine lactoferrin for apoptotic activity. We found that, at higher concentrations, that are present in cows' colostrums, lactoferrin was able to induce apoptosis. Apoptotic activity was detected in a dose as low as 200 ug/mL, within the range naturally present in cow's milk. However, after regression analysis there seems to be a concentration dependent effect of LF; with increasing concentrations there is a coinciding increase with apoptosis. Thus higher concentrations of LF should be tested and may be required for significant apoptotic activity.

Lactoferrin has been shown to inhibit tumour growth and reduced metastasis in lung cancer cells (Bezault et al., 1994), oral administration of LF inhibited 4-nitroquinoline 1-oxide carcinogenesis in rats (Tanaka et a., 2000). Similar apoptotic effects were observed in rats induced by various other carcinogens in esophageal and lung cancers (Ushida et al., 1999; Kuhara et al., 2000), O12 human squamous carcinoma cells (Varadhachary et al., 2004) as well as head and neck cancer cells (Xiao et al., 2004). It is suggested that their effects may be exerted through stimulation of immune production of IL-18, expansion of CD8$^+$ T cells and an enhancement of NK cell lytic activity (Varadhachary et al., 2004) or cellular death via G_0 and G_1 cell cycle arrest (Xiao et a., 2004). Kanyshkova et al. (2003) found that human milk LF contains catalytically active subfractions, which possess DNase, RNase, phophatase and ATPase enzymatic activity, are apoptotic and cytotoxic to mouse fibroblasts and human promyelocytes. In azoxymethane (AOM) induced mice, lactoferrin increased apoptotic activity through upregulation of Fas by inducing fas expression on cell surfaces of NK cells (Fujita et al., 2004). Sakai et al. (2005), found that pepsin digested lactoferrin had significant apoptotic activity against human oral squamous carcinoma cell line SAS, they suggest that milk protein is able to trigger or alter death signaling pathways such as caspase-3 and JNK/SAPK activation. It is also speculated that the iron binding properties of lactoferrin contribute to its antitumour properties, since free iron may induce oxidative damage to DNA (Weinberg, 1996).

These studies provide strong experimental evidence for the use of lactoferrin in the induction of apoptosis; however, in some studies high concentrations of lactoferrin, 2%, were used. This is a concentration not naturally present in cows' milk or colostrums but is in human colostrum and milk. This concentration is physiologically relevant to the neonate, however, for application as a functional food, this concentration is not naturally present in cows milk. Thus there would be increased processing costs involved, whereas at concentration naturally present in milk, no other processing would be required.

Interestingly, several researchers have suggested that it is Lfcin, a peptide fraction located within LF structure, which is responsible for apoptotic activity (Yoo et al., 1997; Eliassen et al., 2002). Indeed, Bovine lactoferricin has been found to induce apoptotic activity in human T leukemic cells via calcium and magnesium dependent endonucleases associated with oxidant dependent death pathway, while lactoferrin was ineffective (Yoo et al., 1997; Mader et al., 2005). Hakansson et al. (1995), found similar results to confirm this. It is suggested that the discrepancy in apoptotic activity between lactoferrin and lactoferricin is due to the exposure state of the active sequence presence in lactoferrin (Yoo et al., 1997). Activation of the mitochondrial dependent pathway of apoptosis has also been implicated in apoptotic mechanism of lactoferricin as destabilization of mitochondrial transmembrane potential results in destabilization of the mitochondrial transmembrane, leading to accumulation of proapoptotic messengers, such as cytochrome c and other proapototic mitochondrial proteins, in the cytoplasmic compartment (Mader et al., 2005). The presence of cationically charged groups within Lfcin, have been implicated in the induction of apoptosis in HL60 human leukemic cells (Roy et al., 2002), since studies have shown that cationic liposomes induces apoptosis, whereas anionic or negatively charged liposome do not have any activity (Aramaki et al., 2000).

Our research demonstrates a marked increase in apoptotic activity from levels naturally present in cow's milk or obtainable from cow's milk (0.2-0.5 g/L). In addition, we found that whole lactoferrin itself had apoptotic activity against human colon cancer cells whereas other researchers found that only pepsin digested lactoferrin had apoptotic activity. It would be interesting to test pepsin-digested lactoferrin for its activity after *in vivo* transit through the gastrointestinal tract. If, pepsin degraded lactoferrin had enhanced activity, this would be a physiologically and commercially ideal situation because milk protein would undergo pepsin/trypsin digestion via intestinal transit to liberate the bioactive peptides such as lactoferricin (Lfcin). However, our results demonstrate that lactoferrin itself possesses apoptotic activity. Thus it remains to be elucidate the precise mechanism with which lactoferrin/lactoferricin exerts action.

We also studied the apoptotic effect of bovine α lactalbumin. α Lactalbumin is secreted by the mammary epithelium and is the major whey protein of human milk (≅2 g/L) (Heine et al., 1991; Shah, 2000) and second highest in cow's milk (≅1.2 g/L) (Saxelin et al., 2003). α Lactalbumin isolated from human milk had apoptotic activity in human lung carcinoma cell line A549, thymocytes, canine kidney cell line, human epithelial cell line (Hakansson et al., 1995) and mouse lymphocytic leukemia cells (Svensson et al., 1999). Studies also show that multimeric α lactalbumin (MAL) isolated from human milk casein, initiate apoptosis in mouse leukemic tumour cells while sparing healthy cells. Its apoptotic effect was attributed to nuclear localization of MAL inducing DNA fragmentation in the nuclei and cleavage of nuclear substrates that are implicated in apoptosis (Hakansson et al., 1999).

HAMLET (human α lactalbumin made lethal to tumour cells), a protein lipid complex, composed of unfolded human α-lactalbumin stabilized by C18:1 cis9 (oleic acid) has been identified as the apoptosis inducing state of α lactalbumin (Svensson et al., 2000; Svensson et al., 2003). HAMLET had apoptotic activity against malignant glioma cells (D54, U251 and CRL2365), while showed sparing activity against non-transformed cells at test concentrations (Fisher et al., 2004); lung carcinoma cells (A549), Jurkat cells, HeLa (Duringer et al., 2003) and lactating mammary glands (Baltzer et al., 2004).

HAMLET binds to histones in tumour cell nuclei resulting in condensation of chromatin structure, a hallmark feature of apoptosis (Duringer et al., 2003; Gustafsson et al., 2005). HAMLET may also activate p53 dependent rescue mechanisms (Fisher et al., 2004).

It is speculated that in the gut of breast fed children, the low pH of gastric juices promotes unfolding of human α lactalbumin and gut lipases cleave fatty acid from milk phospolipases, resulting in the formation of HAMLET. This complex has beneficial implications to neoplastic prevention in breast fed children.

However, it has also been demonstrated repeatedly that native human α lactalbumin lacks apoptotic activity (Hakansson et al., 1999; Baltzer et al., 2004). These studies have concentrated on the use of human milk α lactalbumin, while we concentrated on the use of bovine α lactalbumin. Research performed with human α lactalbumin used concentrations physiologically present in human breast milk (0.3 to 1 mg/mL) however, studies have shown antiproliferative effects of α lactalbumin in concentrations as low as 10-25 ug/mL (Sternhagen and Allen, 2001). We have demonstrated that very small concentrations (10-35 ug/mL) of bovine lactalbumin, which has not undergone conformational changes as seen in HAMLET, were able to prevent cellular proliferation by induction of apoptosis in colon cancer cell line HT29. Indeed, Permyakov et al., found that bovine lactalbumin in its native structure had histone binding capabilities similar to HAMLET (2004). However, whether bovine α lactalbumin interacts directly with nuclear element to induce apoptosis requires further research.

However, the therapeutic possibility of using bovine lactoferrin, that is liberated naturally through digestion or fermentation processes has significant implications to cancer prevention and treatment as well as to the dairy industry. This naturally present bioactive peptide would not require further processing to elicit its apoptotic effects thus represents an economically viable and widely distributed potential anticancer agent.

6.3.2.1 Casein and Casein Derived Proteins

Many studies have been performed on whey protein in cancer, however, there has not been as much focus on casein and casein derived proteins with respect to cancer prevention and treatment. We studied casein hydrolysate, sodium caseinate and β-casomorphin-7 for apoptotic activity in HT29 human colon cancer cells. We found that casein hydrolysate at 40 mg/mL and β-casomorphin-7 (62.5 µg/mL) were able to elicit an apoptotic response.

Casein is the primary protein found in milk (80%). It is the native structure from which many other bioactive peptides have been isolated. Casein itself, however, has not had any biological activities ascribed to it (Meisel and Bockelman, 1999). Indeed, we were unable to detect any apoptotic activity from a sodium salt of casein, sodium caseinate. We were however, able to detect only mild apoptotic activity from hydrolysed casein (Table 6.2).

By definition casein hydrolysate is a mixture of amino acids and peptides produced by enzymatic or acid hydrolysis of casein. The proteolytic treatment of casein could have liberated bioactive peptides from the native structure of casein (as reviewed by Parodi, 2001). Indeed, in rats implanted with Ehrlich ascites tumour cells, yogurt feeding decreased tumour indices; the active ingredient was concentrated in the solid casein fraction of the yogurt (Reddy et al., 1983); Abd El-Gawad et al. (2004), found similar results but the antitumour activity was attributed to the *Bifidobacterium* used in the fermentation. Although these researchers found that when yogurt fermented with *Bifidobacterium* was heated, antitumour activity was significantly diminished. Unfortunately, it was not determined if antitumour activity was due to the death of the bacteria or denaturation of some heat labile protein liberated from milk during fermentation. In cellular uptake studies, CaCo-2 and IEC-6 cells exposed to bacterial hydrolysed casein, demonstrated marked decrease in cellular proliferation, a hallmark indication of cellular damage (MacDonald et al., 1994); *in vivo* tests show similar results (Govers et al., 1993). In dimethylhydrazine treated rats, diets containing casein conferred a significantly higher degree of tumour inhibition than a similar diet with chickpeas as a protein and fiber source; tumour inhibition was attributed to down regulation of secondary bile synthesis and nitrogenous compounds concentration within the intestine (McIntosh et al., 1998). It is thought that caseins confer protection to endothelial cells by inhibiting oxidative reactions through iron chelation and thus iron induced peroxidation and scavenging of free radicals (Cervato et al., 1999; Diaz and Decker, 2004). In our study, we have demonstrated that hydrolysed casein may contribute to cancer prevention or treatment through an alternative mechanism. Perhaps, some bioactive peptide liberated during hydrolysis was able to induce apoptotic death messengers or activate death pathways in cancer cells, to induce apoptosis.

Thus, we studied β-casomorphin-7. β-casomorphin-7, a bioactive peptide liberated from the native structure of casein through enzymatic or proteolytic hydrolysis, has been reported to have a variety of immunologic and anticancer properties (Kampa et al., 1997; Hatzoglou et al., 1996). β-casomorphin-7, is the major exogenous opioid derived from β casein through intestinal chyme or trypsin digestion (Meisel and Bockelmann, 1999). Endogenous or 'typical' opioids are derived from three precursor proteins: pro-enkephalin, pro-dynorphin and pro-opiomelanocortin (Hollt, 1986). Milk derived or 'atypical' opioid peptides do not possess N-terminal amino acid sequence homology with 'typical' opioids. However, they share a common structural feature, the presence of a tyrosine residue at the amino terminal and another aromatic residue in the third or fourth position (Meisel and FitzGerald, 2000).

Opioid receptors and peptides play an essential, yet not understood, role in cancer development and inhibition (Fichna and Janecka, 2004). Studies have found the presence of opioids and opioid receptors in colon cancers (Bostwick et al., 1987; Zagon et al., 1996a; Hytrek et al., 1996). There is accumulating evidence that receptor specific opioids are involved in the induction of apoptosis (Fuchs and Pruett, 1993; Maneckjee and Minna, 1994; Singhal et al., 1998; Goswami et al., 1998). β-casomorphin-7 has been shown to stimulate peripheral blood lymphocytes proliferation (Kayser and Meisel, 1996). In prostatic cancer cell lines, the administration of casomorphins markedly decreased proliferation through interaction with opioid receptors (Kampa et al., 1997). The administration of α- and β-casomorphin in T47D human breast cancer cells lines resulted in decreased cellular proliferation and peptide binding to both opioid receptors and somatostatin receptors (Hatzoglou et al., 1996a; Hatzoglou et al., 1996b). It is hypothesized that binding of opioids by casomorphins to opioid and somatostatin receptors results in the inhibition of adenylate cyclase and by decreasing of intracellular levels of cAMP (Fichna and Janecka, 2004), resulting in cell cycle arrest.

β-casomorphin has been found to inhibit ornithine decarboxylase (ODC) activity in colon cells (Elitsur and Luk, 1991). As previously discussed, ODC is the rate-limiting enzyme involved in polyamine synthesis; decreased polyamine concentration within the luminal environment favours apoptosis. Many cells are resistant to treatment with anticancer agents, however, studies have shown opioid agonist pretreatment of resistant cell lines followed by treatment with anticancer agents significantly enhanced apoptosis. Furthermore, it is suggested that opioids exert their effect by lowering intracellular cyclic AMP levels, through mediation of adenylate cyclase, since opioids/cancer treatment combination exerted the strongest apoptotic effect when intracellular levels of cyclic AMP are at it's lowest (Goswami et al., 1998).

Expression of regulatory molecules Fas/FasL, is associated with proapoptosis. The Fas antigen is a member of the tumour necrosis factor family and induces apoptosis within cells via crosslinking with FasL. Opioid agonist interaction with opioid receptors on cell surfaces has been shown to upregulate Fas/FasL association resulting activation of cellular death signaling. Bcl-2 is a mitochondrial cellular death pathway, which involves the release of cytochrome c into the cytosol and the activation of caspase 9. Bcl-2 family of proteins consists of several proteins that are antiapoptotic (Bcl-2 and Bcl-x_L) or proapoptotic (Bax, Bcl-x_S). Bax antagonizes the antiapoptotic activities of Bcl-2. Treatment of human leukemia cells HL-60 with opioid agonists result in the upregulation of proapoptotic cellular messengers Fas/FasL and Bax expression along with a down regulation of antiapoptotic Bcl-2 (Chatzaki et al., 2001; Lin et al., 2004).

Zagon et al., (1996b) found that HT29 cells treated with opioid growth factor ([Met^5]enkephalin) had decreased proliferative capacity, however, this was not attributed to apoptosis but to cell cycle arrest. Our experimental data shows that bovine β-casomorphin-7 was able to cause cancer cell death via induction of apoptosis in HT29 colon cancer cells. This is an important finding because opioid agonists such as β-casomorphin, naturally present in food, could possibly be important adjuvants in chemotherapeutic modulation of cancer via regulation of cAMP, polyamine synthesis, Fas cellular pathway regulation or via some other regulatory mechanism. However, further research needs to be performed on bovine derived β-casomorphin-7 to determine mode of action and efficacy.

6.2 CONCLUSION

The concept of food having benefits beyond nutrition is extremely attractive from the perspective of both the consumer and the producer. Within North America, in particular, the use of milk associated proteins and bacteria to treat serious diseases, is an attractive postulate because dairy products are readily available, affordable and most importantly well tolerated without any pathogenic effects. Particularly with the increasing prevalence of cancers and increasing cost of health care, this is an extremely attractive proposition. From the dairy producers perspective, milk provides a natural source of therapeutic compounds that do not require further or alteration in existing manufacturing practices to isolate the active ingredients. This study shows that dairy derived LAB and proteins can decrease viability in cancer cells through activation of apoptosis or necrosis. However, this study was only performed *in vitro* using immortalized cancer cell lines, larger animal and clinical studies need to be performed before we can definitively attribute anticancer properties to these compound. Although, these results show promising effects, thus warrant further study.

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Table 6.1. Apoptotic Activity of Dairy Derived Probiotics against Human Colon Cancer Cell Line HT29.

Strain↓/Treatment[c]→	**Apoptotic Activity (Enrichment Factor)[b±]**					
	Live[b]	**Heat Killed**	**24 hour Fermented Milk Supernatant**	**PG1000**	**PG800**	**PG600**
L. acidophilus NRC 13019	1.80 (±0.2)	1.39(±0.4)	1.528(±0.3)	–	–	–
L. acidophilus NRC 13017	3.4(±2.4)	2.6(±0.4)	NA	–	–	–
L. casei ADA 03	2.86(±0.7)	2.40 (±0.8)	NA	2.92(±0.7)	2.05(±0.9)	1.712(±0.2)
L. casei NRC 13005	3.42(±1.2)	2.42(±1.5)	NA	–	–	–
L. acidophilus MR 100	NA	1.90(±0.4)	NA	–	–	–
L. bulgaricus MR 110	NA	1.82 (±0.6)	NA	–	–	–
L. casei CSCC 2601	2.64 (±0.8)	2.08(±0.6)	NA	–	–	–
Yogurt Culture YC085	1.67 (±0.7)	NA	NA	–	–	–

NA: No Activity; -: Not Tested

a. Average value of experiments performed in triplicate

b. In 96 well plates, HT29 colon cancer cells were seeded at 1 × 10^4 cells/well and allowed to attach for 24 hours. The cells were then washed with PBS to remove all non viable cells. Treatments were then added to each well and incubated for 4 hours. Apoptotic activity was measured by the degree of DNA fragmentation, using the Cell Death Detection^{PLUS} assay (Roche Diagnostics, Laval, Quebec, Canada). Enrichment Factor: calculated as treatment absorbance divided by negative control absorbance.

c. None of the treatments were significantly different than control ($p \leq 0.05$).

Table 6.2 Apoptotic Activity of Bovine Milk Derived Protein/Peptides on Human Colon Cancer Cell Line HT29.

Bovine Milk Proteins		Apoptotic Activity
Treatment	Concentration	Enrichment Factor[a,b]
Casein Hydrolysate	40 mg/mL	1.1±1.0
β-Casomorphin-7	62.5 ug/mL	1.89±0.1
Lactoferrin	500 ug/mL	4.12±2.5
	200 ug/mL	1.6±2.2
α Lactalbumin	35 ug/mL	1.247±0.7
	25 ug/mL	1.78±1.1
	10 ug/mL	1.75±1.1

a. Average of 4 experiments performed in triplicate

b. Average value of experiments performed in triplicate b. In 96 well plates, HT29 colon cancer cells were seeded at 1×10^4 cells/well and allowed to attach for 24 hours. The cells were then washed with PBS to remove all non viable cells. Treatments were then added to each well and incubated for 4 hours. Apoptotic activity was measured by the degree of DNA fragmentation, using the Cell Death Detection^{PLUS} assay (Roche Diagnostics, Laval, Quebec, Canada). Enrichment Factor: calculated as treatment absorbance divided by negative control absorbance.

c. None of the data was significantly different than control ($p \leq 0.05$).

Table 6.3. Necrotic Activity of Dairy Derived Probiotics against Human Colon Cancer Cell Line HT29[a].

Strain ↓Treatment→	Necrosis Enrichment Factor[b] Live Cells	Heat Killed
L. acidophilus NRC 13019	20.62±7.97*	5.57±1.77
L. acidophilus NRC 13017	17.37±3.88*	4.60±1.35
L. casei ADA 03	12.20±2.72	2.05±0.42
L. casei NRC 13005	5.09±1.13	NA
L. acidophilus MR 100	NA	0.93±.48
L. bulgaricus MR 110	NA	12.20±6.88
L. casei CSCC 2601	NA	2.74±1.36
Yogurt Culture YC085	10.95±3.81	18.57±14.94*

a. Average of 3-4 experiments all performed in triplicate. Average value of experiments performed intriplicate; NA: No activity

b. In 96 well plates, HT29 colon cancer cells were seeded at 1 × 10^4 cells/well and allowed to attach for 24 hours. The cells were then washed with PBS to remove all non viable cells. Treatments were then added to each well and incubated for 4 hours. Culture supernatants were collected aseptically for necrosis testing. Necrotic activity was measured by the degree of DNA fragmentation, using the Cell Death Detection^{PLUS} assay (Roche Diagnostics, Laval, Quebec, Canada). Enrichment Factor: calculated as treatment absorbance divided by negative control absorbance.

*Significantly different than control ($p \leq 0.05$)

CHAPTER 7 [*]

Immunomodulatory Activity of Milk Derived Bioactive Peptides and Probiotic Bacteria: Possible Biomarkers in Inflammatory Conditions of the Gastrointestinal Tract

7.1 INTRODUCTION

The human gastrointestinal tract is comprised of approximately 400 bacterial species, 30-40 species constituting 99% of the population. Interestingly, *Lactobacillus* and *Bifidobacterium* species are dominant members of these species (Naidu et al., 1999). Lactic acid bacteria (LAB) are now collectively referred to as probiotics. Recently the definition of probiotics, living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Fuller, 1989), has been revised. The definition now proposed for probiotics is, microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being of the host (Salminen et al., 1999). This definition negates the need for live bacteria to be present.

In addition, there are many bioactive peptides that are present in the native structure of milk (Schlimme and Meisel, 1995; Kitts, 1993). These bioactive peptides are liberated during processing or during fermentation by lactic acid degradation or bacterial enzymatic action (Gatti et al., 2004; Law and Haandrikman, 1997). The major proteins present in milk are casein and whey. Within the protein structure of casein and whey are bioactive proteins and peptides, such as α-lactalbumin, β-lactoglobulin and β-casomorphin.

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Many health benefits have been conferred to LAB and milk derived bioactive peptides including treatment of rotoviral infections (Szajewska et al., 2000), atopic dermatitis (Kalliomaki et al., 2001), alteration of gut flora (Romond et al., 1998) and treatment of diarrhea (Marteau et al., 2001). Furthermore, LAB and dairy derived milk proteins/peptides administration has been associated with antitumour activities (Rao et al., 1999; Gallaher et al., 1996; McIntosh et al., 1999; Macleod et al., 1995; Targen et al., 1997). LAB has also been shown to have a beneficial effect in the treatment of inflammatory bowel disease (IBD; Borody et al., 2003; Guslandi et al., 2000; Gionchetti et al., 2003; Laake et al., 2003). It is generally thought that probiotics exert their effects through alteration of intestinal flora, competition of adhesion sites (Bengmark, 1998) and nutrients between the ingested bacteria and potential pathogens (Bernet et al., 1994), production of antibacterial substances and the action of these bacteria (Barefoot and Klaenhammer, 1984) or alteration of bacterial enzymes (Perdigon et al., 1988).

Although there have been many observed beneficial effects of probiotic bacteria and dairy derived milk proteins/peptides as adjuvants in the treatment of various digestive diseases, the mechanisms involved in the improvement of inflammatory conditions associated with digestive diseases are not completely understood and are most likely complex in nature. Recently, focus has shifted to the immunomodulatory effects of probiotics (Tejada-Simon and Pestka, 1999; Dotan and Rachmilewitz, 2005; Neish et al., 2000).

The immune system is a very dynamic system that is kept in delicate balance by proinflammatory and anti-inflammatory processes. Dysregulation of the immune system can result in the manifestation of a variety of disorders including IBD and cancer. Inflammatory bowel disease, consists of Crohn's disease (CD) and ulcerative colitis (UC), is defined as a chronic inflammation of the gastrointestinal tract (Atreya and Neurath, 2005), which is mediated by inflammatory cytokines (Fiocchi, 1998). Furthermore, chronic inflammation can lead to the development of colorectal cancer, a disease also mediated by the action of inflammatory cytokines.

Tumour necrosis factor alpha (TNF α) and interleukin 6 are pleiotropic cytokines with powerful and wide ranging effects upon the immune system. In IBD unrestrained activation of both of these inflammatory cytokines seems to play a pivotal role on the pathogenesis of altered mucosal immune function (Van Deventer, 1997; Atreya and Neurath, 2005). Research on modulation of interleukin 6 (IL 6) is relatively in its infancy. Only recently has there been evidence suggesting IL 6 has been positively correlated with the pathogenesis of colon cancer. Schneider et al (2000) demonstrated that IL 6 stimulated the growth of human colon cancer cells *in vitro*; several carcinoma cell lines secrete IL-6 (Basolo et al., 1996; van Meir et al., 1990; Watson et al., 1990). Moreover, IL 6 serum concentration in patients with colorectal cancer are dramatically elevated and positively correlated with progression of disease and mortality (Chung and Chang, 2003). Indeed, in patients with CD serum IL 6 levels are also elevated (Holub et al., 1998) and have been positively correlated with inflammation (Reinisch et al., 1999). Furthermore, the administration of anti-interleukin 6 monoclonal antibodies in murine models of experimental colitis prevented disease associated wasting and inhibited development of lesions (Ito, 2003).

TNF is considered to be a key inflammatory cytokine involved in the dysregulation of IBD and cellular growth in cancer. TNF is a pivotal player in cellular proliferation, differentiation and apoptosis, as well as inducing other cytokines and immunoregulatory mediators (Papadakis and Targan, 2000). In Crohn's disease, TNF α up regulates many inflammatory mediators, activates neutrophils, macrophages and stimulates B cells. The inhibition of TNF α production in IBD has promising therapeutic benefits in the treatment of Crohn's disease (Lakatos, 2000). Indeed, the inhibition of *Helicobacter hepaticus* induced IBD, was achieved with the administration of *Lactobacillus casei,* which down regulated TNFα secretion (Pena et al., 2005).

The aim of this study was to isolate dairy derived milk proteins/peptides and LAB that have immunomodulatory effect on TNFα and IL-6 production. In addition, we wanted to determine if a cocktail of the eight strains tested had further effects on TNFα/IL-6 production, through synergy between the several bacteria. The effects could possibly then be applied into a dairy based functional food product with immune altering capabilities both for prevention in healthy individuals or as an immunoadjuvant to treatment in GI disorders.

7.2 MATERIALS AND METHODS

7.2.1 Raw 264.7 Mouse Macrophage Cell Line Propagation and Growth

Murine macrophage cell line RAW 264.7 (ATCC: TIB-71) was kindly provided by Luis Hildalgo from Dr. Halloran's laboratory (Medical Microbiology and Immunology at the University of Alberta, Edmonton, Alberta, Canada). RAW 264.7 cells were grown according to American Type Culture Collection (ATCC) recommendations. Briefly, RAW 264.7 cells were grown in Dulbecco's Minimal Essential Media (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1.5 g/L of sodium bicarbonate (Sigma-Aldrich), 10% (v/v) FCS (Gibco Laboratories, Chagrin Falls, IL, USA), 100 U/mL penicillin, 100µg/mL streptomycin and 100 U/mL amphotericin (Sigma-Aldrich). Cells were grown to 80% confluence at 37° C in 5% CO_2/95% air in 75 cm^2 vented tissue culture flasks (Corning, Fisher Scientific, Nepean, ON, Canada). Cell number and viability were assessed using trypan blue exclusion with a haemocytometer. DMEM was changed every second day to third day. Cell detachment was achieved via trypsinization (Sigma-Aldrich) or cell scraping.

RAW 264.7 murine macrophage cells were seeded at a concentration of 1×10^6 cells/well in a 96 well plate (Corning, Fisher Scientific). These cells were allowed to attach for 24 hours. The cells were washed 5 times with fresh DMEM (without antibiotic/antimycotic) to remove all residual amounts of antibiotic. Then 100 µL of *Salmonella typhimurium* LPS (1 µg/mL final concentration; Sigma Aldrich) dissolved in DMEM was added to each well to activate the macrophages. Then 100 µL of the bacterial samples were added to each well. *Salmonella typhimurium* LPS was used as a positive control (100 µL/well). The plates were then incubated at 37°C in 5% CO_2 for 48 hours. After incubation, the plates were centrifuged at 3000 rpm (Accuspin Micro, Fisher Scientific, Nepean, ON, Canada) for 10 minutes (Ring et al., 2000) and the supernatants collected were used immediately for cytokine testing. Each assay was performed in triplicate.

7.2.2 Probiotic LAB Propagation and Growth

Eight strains of lactic acid bacteria, *Lactobacillus acidophilus* MR 100 (MR 100), *Lactobacillus bulgaricus* MR 110 (MR 110), *Lactobacillus acidophilus* NRC 13017 (NRC 13017), *L. acidophilus* NRC 13019 (NRC 13019), *Lactobacillus casei* ADA 03 (ADA 03), *Lactobacillus casei* NRC 13005 (NRC 13005), *Lactobacillus casei* subsp. *Casei* CSCC 2601 (CSCC 2601) and Yogurt Culture YC 085 (YC085) were used in this experiment. In addition, a combination of these eight strains was grown together in one flask (total concentration of 1% inoculum). These strains were grown in MRS broth (Difco) at 37°C for 24 hours (bacterial cell density 1×10^9 cells/mL). The cells were then washed using phosphate buffered saline (PBS). After each wash, cell suspension was centrifuged at 10,000 g for 2 minutes to collect cells. Cell wash was repeated three times. The bacterial cells were then resuspended into DMEM (10% FCS) without antibiotic/antimycotic immediately prior to usage. The bacterial strains were stored at -70°C in MRS broth containing 20% glycerol.

The previously mentioned eight single strains of LAB and the mixture of LAB were heat killed. The cells were washed three times in PBS as described above. The cells were then placed into a 60°C water bath and incubated for 60 minutes. The heat-killed cells were then centrifuged (10,000 g for 2 minutes), resuspended into DMEM (10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 100 U/mL of amphotericin; Sigma Aldrich), and stored at -20°C until used.

7.2.3 *L. casei* ADA 03 Peptidoglycan Isolation and Purification

Peptidoglycan was isolated from *L.casei* ADA 03 according to the methods reported by de Ambrosini et al., (1996). Briefly, resuspended cells of *L.casei* ADA 03 in PBS were subjected to mechanical disruption using a French Press. Broken cells were centrifuged (10,000 g) for 10 minutes and the pellet obtained was suspended in 4% boiling sodium dodecyl sulphate (SDS; Sigma Aldrich) in order to dissolve the cell wall fraction. After 18 hours of incubation, the suspension was centrifuged at 20, 000 g for 20 minutes, and washed with PBS to remove SDS. The pellet was then treated with RNase and DNase to remove nucleic acids, followed by washing with PBS. The pellet suspended in PBS was then treated with trypsin to digest any cell wall associated proteins. This preparation of cells was then washed with PBS and treated with 2% SDS for 4 hours to remove all proteins. Then the sample was washed thoroughly with PBS and deionized water (Milli Q, Millipore Corporation, Concord, CA, U.S.A.). This fraction was considered as the whole cell wall preparation.

The insoluble cell wall precipitate was then treated with 10% trichloroacetic acid (Sigma Aldrich) for overnight period to remove teichoic acid from in the cell wall. The suspension was centrifuged and the pellet was washed thoroughly with PBS and Milli Q water. This pellet was taken as peptidoglycan. For experimental testing, the pellet was suspended in McCoys Modified 5A medium.

7.2.4. Milk Derived Bioactive Protein/Peptide Preparation

Sodium caseinate, β-lactoglobulin, lactoferrin, α-lactalbumin, casein hydrolysate and β-casomorphin-7 were all purchased from Sigma Aldrich (St. Louis, MO, USA). The following concentrations were used for each protein/peptide fraction: sodium caseinate: 75, 37.5 and 18.75 mg/mL; β-lactoglobulin: 120, 90, 60, 30, and 15 mg/mL; lactoferrin: 500, 200, 100, 20 μg/mL; α-lactalbumin: 35, 25 and 10 μg/mL; casein hydrolysate: 40, 20 and 10 mg/mL and β-casomorphin-7: 125, 62.5 and 31.25 μg/mL. Each protein/peptide solution was made in DMEM supplemented with 1.5 g/L of sodium bicarbonate and 10% FCS and filter sterilized with a 0.25 μm syringe filter disc (Acrodisc, Gelman Sciences, Ann Arbor, MI, U.S.A.). The sterile solutions were made immediately prior to cytokine testing.

7.2.5 RAW 264.7 and Bacteria/Protein/Peptide Incubation

RAW 264.7 murine macrophage cells were seeded at a concentration of 1×10^6 cells/well in a 96 well plate (Corning). These cells were allowed to attach for 24 hours. The cells were washed 5 times with fresh DMEM (without antibiotic/antimycotic) to remove all residual amounts of antibiotic. For cytokine testing on stimulated macrophages, 100 µL of LPS dissolved in DMEM (1 µg/mL final concentration) was added to each well except the background and negative controls. Bacterial or protein/peptide samples (100 µL) were added to each well. For cytokine testing on unstimulated macrophages 100 µL of LPS dissolved in DMEM (1µg/mL) was added only to the positive control. The plates were incubated at 37°C in 5% CO_2 for 24 hours before supernatant was collected.

To collect the supernatant, the plates were centrifuged at 3000 rpm for 10 minutes. The supernatants were collected and stored at -20°C until used for cytokine evaluation. Each experiment was performed in triplicate.

7.2.6 Cytokine Elisa Testing

7.2.6.1 Tumour Necrosis Factor (TNF α) and Interleukin 6 (IL-6)

Samples studied in this experiment included: probiotic LAB (live and heat killed), peptidoglycan isolated from *L.casei* ADA 03, supernatants from 24 or 48 hour fermented milk and milk derived proteins/peptides (see above). The experiment was undertaken to evaluate the effects of these samples on the expression of cytokines, specifically TNF α and IL 6 in RAW 264.7 murine macrophage cell line. Two commercially available kits, BD OptEIA Mouse TNF (Mono/Mono) and BD OptEIA Mouse IL 6 ELISA sets (BD Biosciences Pharmingen, Mississauga, ON, Canada) were used. Testing was performed according to the supplier's set protocol. Each assay was performed in triplicate.

7.2.7 Calculation of Cytokine Concentration

The mean absorbance was calculated from the triplicate absorbance values obtained in each assay. This value was then compared and absorbance value obtained from the standard curve for each cytokine to calculate the concentration of TNF α or IL-6 in the spent culture supernatants.

7.2.8 Statistical Analysis

Statistical Analysis was carried out in the Statistica program (Statistica 6.0). The data was analyzed through analysis of variance, Tukey's test, correlation of analysis and regression analysis.

7.3 RESULTS AND DISCUSSION

7.3.1 TNF α Stimulatory Activity of Dairy Derived Protein/Peptides and LAB

We tested various strains of LAB (Table 7.1) and dairy derived milk proteins (Table 7.2) for their ability to stimulate the production of TNF α in RAW 264.7 mouse macrophage cells. We found that the heat killed cells and peptidoglycan were able to stimulate TNF α production to various levels. The most potent stimulation was observed in HK MR 100, it significantly ($p \leq 0.05$) stimulated the production of TNFα to nearly 50% that of the positive control; heat killed NRC 13005, MR 110 and NRC 13019 also significantly ($p \leq 0.05$) stimulated the secretion of TNFα from unstimulated mouse macrophage cells. The remaining strains stimulated between 8-35% that of the positive control. Interestingly, we found that peptidoglycan isolated from ADA 03 had slightly, however statistically insignficant, stronger stimulatory activity than the heat killed cells, perhaps identifying the peptidoglycan as the active component in TNF α stimulation. Although some researchers have noted the TNF α stimulatory activity of live bacteria (Rangavajhyala et al., 1997; Miettinen et al., 2000; Haza et al., 2004; Haza et al., 2005) we did not observe similar stimulatory effects by the live strains we tested (results not shown). The lack of activity observed with the live cells could be due to the point in which the bacteria were harvested; we harvested our bacteria during the stationary phase of growth. Live bacteria harvested in the exponential phase have been shown to have stronger TNFα stimulatory activity, than others harvested during other phases; in fact heat killed lab are more effective inducers of TNF production when harvested in the stationary phase (Haller et al., 1999; Wallace et al., 2003).

It is speculated that soluble mediators and bioactive proteins/peptides that are released into the growth medium during fermentation by LAB are partially responsible for immunomodulatory activity (Foucaud and Juillard, 2000; Matar et al., 2001; Law and Haandrikman, 1997; Madsen et al., 2001; Menard et al., 2004). Indeed, Kitazawa et al., (2002) isolated from *L. gasseri* a soluble mediator termed Gasserokine, that has chemotactic/activation effects on both murine and human macrophage accumulation at the sight of injury; while, LeBlanc et al., (2002) isolated peptide fractions from *L. helveticus* R389 fermented milk that had growth inhibitory effects on fibrosarcoma tumours. In addition, lysozyme that is found in the body can cleave bacterial cell walls, releasing absorbable fragments of peptidoglycan that then can have immunomodulatory effects (Hatcher and Lambrecht, 1993; Menard et al., 2004).

In fermented milk, enzymatic or acid digestion of the native protein structure, liberates bioactive proteins/peptides, such as β-casomorphin, lactoferrin, β-lactoglobulin, α lactalbumin. Indeed, our results demonstrate that these individual fractions have, albeit mild as compared to bacterial cell wall, TNFα stimulatory activity (Table 7.2). Our results demonstrate that casein hydrolysate, a mixture of amino acids and peptides produced by enzymatic or acid hydrolysis of casein, has TNFα stimulatory activity. Furthermore, β-casomorphin, an opioid peptide isolated from casein, has TNF α stimulatory activity. However, none of these treatments were significantly different than the control. In addition, we found that soluble whey proteins lactoferrin and α lactalbumin also had TNFα stimulatory activity. Lactoferrin at 10 μg/mL had significant TNFα stimulatory activity. Furthermore, it is interesting to note that at higher concentrations of α lactalbumin (>10 μg/mL) activity was lower than at 10 μg/mL, indicating possibly the activation of a negative feed back loop at higher concentrations. Thus indirectly showing that bacterial fermentation results in liberation of bioactive peptides.

However, from our results we were unable to observe any stimulatory activity from the fermented milk samples (data not shown), which is interesting because as previously discussed several of the individual proteins found in the soluble fraction, had stimulatory activity, albeit mild (Table 7.2). This could be due to the presence of some inhibitory, yet unidentified, compounds in the fermented milk, since fermented milk samples significantly ($p \leq 0.05$) inhibited TNFα production in activated macrophages; or perhaps the different enzymes produced by the eight strains of bacteria hydrolyzed protein/peptide fractions to smaller fractions that are no longer able to mediate TNFα production (Law and Haandrikman, 1997). Our results are in line with Pena and

Versalovic (2003) who found that bacterial conditioned media did not stimulate TNFα from naive RAW 264.7 cells; however, with *S. thermophilus* conditioned media (bacteria free), in the absence of any other stimulation *S. thermophilus* conditioned media stimulated TNFα secretion (Menard et al., 2004).

Researchers have found that heat killed cells are potent stimulators of TNF α secretion. Rangavajhyala et al. (1997), found several strains of *L. acidophilus* stimulated TNF α production from macrophages. The strains we tested had dramatically higher stimulatory activity on macrophage cells. *L. acidophilus* LA1 stimulated macrophages to produce 588 pg/mL of TNF (Rangavajhyala et al., 1997), while the majority of our strains produced TNF levels dramatically higher (Table 7.1). Furthermore, others have demonstrated that exposure of heat killed cells of *Bifidobacterium* Bf-1, *L. acidophilus* La-2, *L.bulgaricus* 1489 NCK 23, *L.casei* ATCC 39539, *L. gasseri* ADH NCK 101, *L. helveticus* Lr-92, *L. reuteri* ATCC 23272 and *S. thermophilus* St-133 had significant stimulatory activity greater than that found in LPS stimulated macrophages (Tejada-Simon and Pestka, 1999). Indeed, Matsuguchi et al. (2003) found that several strains of *Lactobacillus* (*L. casei* YIT 9029, *L. fermentum* YIT 0159, *L. rhamnosus* YIT 0232, *L. acidophilus* YIT 0070, *L. plantarum* YIT 0102 and *L. reuteri* YIT 0197) had significant stimulatory activity on mouse mononuclear cells, producing nanograms of TNF α. *In vivo*, intrapleural injection of *Lactobacillus casei* strain Shirota (LC 09018) showed significant antitumour effects. This antitumour activity was attributed to the induction of TNF α secretion from macrophages; this antitumour activity was completely abrogated with co-administration anti-TNF monoclonal antibodies (Yatsutake, et al., 1999).

In general, from the present study all the heat killed strains induced TNF α secretion from macrophages; the degree of stimulation was varied. This observation is consistent with previous reports on the proinflammatory cytokine potentiating activity of LAB (Maasen, et al., 2000; Marin et al., 1998; Matsuguchi et al., 2003). However, in activated macrophages, several of the heat killed samples significantly ($p \leq 0.05$) augmented the production of TNF α beyond that achieved by the LPS control (Table 7.4), where TNFα concentrations were measured at nanogram levels. This leads us to speculate that the potency of the heat killed LAB is enhanced when the macrophages have been activated. The implications of this hyper induction are great. In many cancer cells, there is resistance to cytotoxic activity of circulating immune cells (Chouaib et al., 2002), perhaps, the elevated response induced by some of our bacterial species can augment the cytotoxic action of the immune cells.

It is hypothesized that much of the activity attributed to LAB are due to the cell wall components, specifically lipoteichoic acid and peptidoglycan (Okamoto et al., 2002). Our results show that peptidoglycan isolated from ADA 03 had moderate, but statistically insignificant, stimulatory activity in unstimulated mouse macrophages, which are in line with results from Matsuguchi et al., (2003). However, in LPS stimulated cells peptidoglycan activity was divergent depending upon concentration. Moderate inhibitory activity was observed at 800 and 600 μg/mL (Table 7.3), whereas, at 1000 μg/mL, there was significant stimulatory activity in activated macrophages (Table 7.4). This dosage effect could have important therapeutic implications, depending upon the desired response, the required dose is chosen accordingly.

Since four of the eight LAB tested had profound effects on the stimulation of TNFα, we tested a cocktail of all the eight strains in hopes of developing a cocktail of bacteria that acted synergistically to augment immunomodulatory action. The heat killed mixture induced mild stimulation of TNFα production from unstimulated macrophage cells, however, the degree of stimulation was slightly lower than several of the individual treatments (Table 7.1). Furthermore, in macrophage cells activated with LPS there was also significant augmentation of TNFα production from several individual heat killed strains (Table 7.4), however, with the heat killed mixture of all the strains, this activity was lower than the individual cells. Taken together, this suggests that there is an antagonistic effect of individual bacteria against each other. Similarly, Perdigon et al. (1991), found that co-administration of *L. acidophilus* and *L. casei* did not augment antitumour activity, in fact, similar to our results, there was a down regulation of activity as compared to individual strains. Yet, Fukui et al. (2001), found the co-administration of LDS (*Lactobacillus* dietary supplement; *L. casei subsp. tolerans, L. acidophilus, L.*

fermentum and *L. rhamnosus*) had significant inhibitory DMH induced tumour progression in mice. However neither was able to elucidate the mechanism of action. This underlies the profound differences in biological activities between and within strains of LAB.

7.3.2 TNF α Inhibitory Activity of Dairy Derived Protein/Peptides and LAB

Recent studies in the treatment of IBD have been aimed towards altering cytokine expression, specifically down regulating proinflammatory cytokine TNFα. The drug infliximab decreases TNF concentrations *in vivo* by directly binding to TNFα. IBD patients treated with infliximab, reached and maintained remission for periods longer than that of the control, without requiring steroidal treatment (Scholmerich and Huber, 2003). Others have shown improvement in disease state by the use of anti-TNF monoclonal antibodies (Van Dullemen, et al., 1995; Stack et al., 1997; Targan et al., 1997). In addition it is suggested that an imbalance between tumour necrosis factor and its inhibitor, soluble tumour necrosis factor receptors (sTNF-R1 and sTNF-R2) *in vivo*, is perhaps a contributing factor to the pathogenesis of IBD. Indeed, there was a significantly increased concentration of TNF in involved Crohn's disease and ulcerative colitis tissue as compared to uninvolved neighboring tissue, however, there was not a concomitant increase in sTNF-R (Noguchi et al., 1998). This increased expression of TNF was significantly correlated with severity of disease (Borruel et al., 2002). The administration of inhibitor sTNF-R monoclonal antibody abrogated all protective effects of sTNF-R; function was restored after administration of human sTNF-R (Noguchi et al., 1998). Such findings have lead to the search for other substances that can inhibit or down regulate TNF production.

In the present experiment, we have found that for all the eight strains, the live and the fermented milk supernatants had statistically significant inhibitory activity against LPS activated RAW 264.7 mouse macrophage cells (Tables 7.3). Interestingly, all the heat killed cells, with the exception of ADA 03 and NRC 13017, had significant ($p \leq 0.05$) stimulatory activity. Several of the strains stimulated the secretion of TNF α at the nanogram concentration. The hyper stimulation by the heat killed cells could be due to the stage that we harvested the bacterial cells. We harvested the bacterial cells during the stationary phase; studies have demonstrated that heat killed, stationary phase bacteria are significantly more potent TNF α stimulators than heat killed bacteria harvested during the exponential phase (Haller et al., 1999). Contrary to our findings, Wallace et al. (2003) reported that *L. rhamnosus* R0011, *B. longum* R015 and *L. delbrueckii* R0187 all had down regulated TNF α secretion; Borruel et al. (2002) did not observe any change in activity by heat killed *L. casei* or *L. bulgaricus*. Regardless, these findings have profound implications for the use of probiotics as immunomodulatory agents, in particular for immunosuppressed individuals where live bacteria supplementation is problematic.

The live and the fermented milk supernatants had significant ($p \leq 0.05$) inhibitory activity. All of the live bacterial treatments tested had significant inhibitory activity (Table 7.3). This is in line with results from other researchers. Borruel et al. (2002), found that *L casei and L. bulgaricus* significantly decreased TNFα secretion by inflamed colonic tissue isolated from Crohn's' disease patients whereas in normal tissue, there was no effect. It is interesting to note that the fermented milk supernatants were just as effective as the live treatments to suppress TNF secretion. This suggests that during fermentation through bacterial enzymes or acid degradation, immune modulating substances, such as immunomodulins or proteins/peptides are liberated from milk. These soluble substances have the ability to modulate immune function without the requirement for live bacterial cells. This is important to the dairy industry it eliminates the need for live cells, as viability of LAB due to shelf life and storage conditions has been a topic of controversy. In addition, the possible side affects associated with live bacterial cell supplementation, such as sepsis or bacteremia (De Groote et al., 2005; Land et al., 2005; Salminen et al., 2002); or fever and malaise, commonly associated with bacterial treatments (Patard et al., 2003; Lockyer and Gillat, 2001) could possibly be circumvented.

Although, we cannot directly identify the presence of immunomodulins, indirectly, through testing of various protein/peptide fractions we have demonstrated that whey proteins, β-lactoglobulin, lactoferrin and α-lactalbumin had concentration dependent inhibitory activity (Table 7.5). At all concentrations of sodium caseinate, β-lactoglobulin and lactoferrin tested, there was a statistically significant ($p \leq 0.05$) inhibition of TNFα secretion. With casein hydrolysate at the highest concentration tested, there was significant ($p \leq 0.05$) inhibitory activity, whereas at the lowest concentration tested there was significant ($p \leq 0.05$) stimulatory activity. With β-casomorphin-7 significant ($p \leq 0.05$) inhibition was observed only at the highest concentration tested (125 ug/mL), however at lower concentrations TNF α secretion was still inhibited by greater than 50%. Interestingly, with α-lactalbumin the highest, although statistically insignificant, degree of inhibition was observed at the lowest concentration; degree of inhibition decreased with increasing concentrations.

During fermentation, either through bacterial enzymatic action or lactic acid digestion of milk proteins, these proteins and peptides are liberated into the soluble whey protein fraction or in our experiment, the fermented milk supernatant (Foucaud and Juillard, 2000; Matar et al., 2001; Law and Haandrikman, 1997). Thus it could be through the action of probiotic fermentation that TNFα inhibitors are liberated from milk. Furthermore it has been suggested that fermented milk supernatant could also contain small amounts of peptidoglycan from LAB (Law and Haandrikman, 1997). Our results support this finding because peptidoglycan from ADA 03 had moderate TNF α inhibitory activity.

Pena and Versalovic (2003) found similar results using bacterial conditioned medium (cm). They found that *Lactobacillus rhamnosus* GG-cm had significant TNF α inhibitory activity in LPS activated macrophages. In addition, Menard et al. (2004) found that conditioned media from probiotic strains, *Bifidobacterium breve* and *Streptococcus thermophilus* had significant inhibitory activity in LPS stimulated immune cells. In addition, they found that pepsin-trypsin hydrolysis did not decrease the inhibitory activity of the conditioned media, suggesting that the active metabolites are not proteinaceous. Conversely, Madsen et al. (2001) suggested that a proteinaceous soluble factor secreted by probiotic bacteria preparation VSL# 3 helped improve epithelial barrier function in IL-10 knockout mice. However, from our results we cannot conclude whether it was a proteinaceous or non-proteinaceous soluble mediator release by LAB that contributed to TNF α suppression.

Menard et al. (2004) suggest that bacterial metabolites may inhibit LPS activity through the following mechanisms; a) limited access to CD14 receptors on monocytes/macrophages. Limited access of LPS to CD14, results in a lowering of nuclear factor kappa-B (NF-KB) activation and consequent immune cell signaling that down regulates TNF secretion and b) LAB are able to stimulate potent anti inflammatory cytokine IL-10 secretion by macrophages (Pathmakantahn et al, 2004; Menard et al., 2004). IL-10 down regulates TNF α secretion by macrophages (Fiorentino et al., 1991).

Since the live and fermented milk supernatants, had such significant inhibitory activity on TNF α secretion, we wanted to develop a cocktail of bacteria that could possibly have synergistic activity in immunomodulation (Table 7.3). The cocktail of the bacterial strains also had significant inhibitory activity against TNFα secretion from mouse macrophages. The inhibitory activity was not as high as that observed with the individual strains, suggesting that there was antagonistic activity occurring. It has been shown that co-culture of different strains of bacteria result in inhibitory activity of one strain against another strain (Christensen, et al., 2002). Alternately, the observation that individual strains had a similar degree of inhibition as the cocktail perhaps is an indication that synergistic activity is not required, the individual strains are powerful enough inhibitors. However, we cannot discount the effect of a highly concentrated mixed LAB cocktail, as seen in VSL#3, to have profound immunologic effects. Further experiments need to be performed.

Indeed, in animal studies, several strains of LAB had the ability to improve IBD pathogenesis. Pena et al.(2005) studied the effect of probiotic strains *Lactobacillus paracasei* 1602 and *Lactobacillus reuteri* 6798 on *Helicobacter hepaticus* 3B1 induced colitis in IL-10 knock out mice. IL-10 knock out mice are considered excellent animal models for the study of human colitis (Kullberg et al., 1998). They found that co-administration of *L. reuteri/L. paracasei* resulted in significant reduction in *H. hepaticus* mediated production of TNFα. In addition, in animals co-treated with these two probiotic strains there was a significant diminuation of IBD-like colon lesions (Pena et al., 2005). Using the same mouse model, Madsen et al. (2001), found that probiotic preparation VSL#3 significantly inhibited TNFα secretion. In fact other researchers found that mice treated with anti-TNFα monoclonal antibody, had vast improvements in colitis (Gratz et al., 2002). Furthermore, treatment with individual strains of bacteria has resulted in attenuation of colitis in mice through down regulation of inflammatory cytokine TNF α (McCarthy et al., 2003).

7.3.3. The Stimulatory Effect of Dairy Derived LAB and Milk Proteins/Peptides on Inflammatory Cytokine Interleukin 6

We examined the effect of the eight strains of LAB and milk proteins/peptides on interleukin 6 secretion in LPS stimulated and unstimulated RAW 264.7 mouse macrophage cells. We observed induction of only mild stimulatory activity in the naive unstimulated mouse macrophages by LAB and milk proteins. Statistically significant stimulation of IL 6 secretion was induced by all heat killed cells with the exception of NRC 13017 and CSCC 2601 (Table 7.6). Furthermore, all concentrations tested for ADA 03 peptidoglycan significantly induced secretion of IL 6 from naive mouse macrophages. β-lactoglobulin and lactoferrin stimulated macrophage secretion of IL 6 but not at a statistically significant level, however, in the treatments tested, other than those mentioned, secretion was negligible as compared to the control. The live cells nor fermented milk samples had any observable activity

These results are in line with other researchers. Morita et al. (2002), found several heat killed LAB of dairy and human origin induced IL-6 secretion in J774.1 murine macrophages. Ruiz et al. (2005) observed that *Bifidobacterium lactis* was able to transiently increase IL-6 secretion from intestinal epithelial cells. The administration of *L. reuteri* and *L.casei* individually up regulated secretion of IL-6, along with other pro-inflammatory cytokines from dendritic cells (Christensen et al., 2002). This is an important finding, as dendritic cells have been shown to have the ability to penetrate the epithelium without disrupting the barrier function and directly sample indigenous or probiotic bacteria within the gut (Roscigno et al., 2001). Miettinen et al. (2000) observed that the administration of live *L.rhamnosus* GG induced the secretion of IL-6 in RAW 264.7 macrophage cells. *L. rhamnosus* induced secretion of IL-6 to nanogram concentrations after 24 hours of incubation. It is interesting to note that *L. rhamnosus* stimulation was similar to that of major Gram-positive human pathogen *Streptococcus* pyogenes. However, *in vivo* studies have demonstrated, as compared to endogenously derived strains from healthy donors, oral administration of live cultures of dairy derived LAB (*L. fermentum*) did not stimulate secretion of similar levels of IL-6 (Vinderola et al., 2004). Duc et al. (2004), observed similar elevations of IL-6 secretion by administration of *Bacillus* species.

In addition, we found that a cocktail of the dairy strains induced the secretion of IL-6 from mouse macrophages ($p \leq 0.05$) (Table 7.6). However, levels of IL-6 secreted were not as high as the individual strains, indicating antagonistic activity between strains or some substance produced/released during heat killing exert an antagonistic effect upon each other. However, other researchers have encountered the phenomenon (Viljanen et al., 2005a; Viljanen et al., 2005b). In the present study, the heat killed bacterial strains tested were able to induce mild, yet statistically significant, secretion of IL 6. This low level of stimulatory activity may be physiologically significant as there is a delicate balance between pro-inflammatory and anti-inflammatory cytokines; severe perturbation in either direction could result in development or exacerbation of disease.

It is interesting to note that peptidoglycan isolated from ADA 03 showed the strongest stimulatory activity at 800 or 600 µg/mL (2229 pg/mL and 2471 pg/mL), whereas at 1000 µg/mL activity was slightly lower (1400 pg/mL). This could possibly be due saturation of limited peptidoglycan binding sites on macrophages (Dziarski, 1991). It has been shown that peptidoglycan stays in tissues for longer periods of time than other active components (Dziarski, 1982). This could be therapeutically significant and manipulated to achieve the desired therapeutic outcome.

Thus, therapeutic potential of LAB with stimulatory activity on proinflammatory cytokine IL 6 secretion exists. Treatment of children with IgE mediated atopic eczema-dermatitis syndrome with probiotic *L. rhamnosus GG* have resulted in significant improvement of eczema (Viljanen et al., 2005a). This improvement was attributed to increased serum levels of IL 6 to dietary supplementation with *L. rhamnosus* GG. This suggests that stimulation of the immune system and low-grade inflammation induction by probiotic administration may be of therapeutic potential in allergic children (Viljanen et al., 2005b). In fact, serum concentrations of IL 6 induced by *L. rhamnosus* GG were similar to those observed in our *in vitro* experiments. Indeed, in healthy adults, administration of IL 6 induced an anti-inflammatory response (Steensberg et al., 2003).

7.3.4. The Inhibitory Effect of Dairy Derived LAB and Milk Proteins/Peptides on Inflammatory Cytokine Interleukin 6 Secretion

Conversely, in the LPS activated cells, we notice a significant inhibition by all strains and treatments of bacteria (Table 7.7). There is significant inhibition of IL 6 by live bacteria and fermented milk supernatants, reducing secretion back to the level of the negative control. Although the heat killed and peptidoglycan fractions did not induce complete inhibition, there was still significant inhibition observed. Inhibitory activity for the heated killed cells and peptidoglycan ranged from 75.41% (ADA 03 peptidoglycan 1000 μg/mL) to 94.99% (NRC 13017).

It is interesting to note that Dziarski (1982) observed that bacterial cell wall components remained present in tissue for long periods of time. This is promising for our results because the peptidoglycan, heat killed fractions and the fermented milk supernatants all had significant inhibitory activity. The process of heat killing and fermentation of milk supernatants could have liberated cell wall fragments that have immunopotentiating activity. Thus these are potential candidates for prolonged therapeutic activity.

Our results are in line with other researchers. Matsumoto et al. (2005) found that *L casei* strain Shirota, significantly inhibited IL-6 secretion from RAW 264.7 cells, lamina propria mononuclear cells and tissue for patients with inflamed colons. In addition, they found that treatment with cell wall fractions induced similar reductions in IL-6 in the same systems. However, the percentage inhibition achieved by all of our experimental strains was dramatically higher, than those found by Matsumoto (maximum inhibition: 80%; 2005). The administration of *E.coli* strain Nissle 1917 to colitic SCID mice down regulated IL-6; it is this inhibition of pro-inflammatory cytokine IL-6 that is thought to contribute to improvement in colitis disease parameters (Schultz et al., 2004; Atreya and Neurath, 2005). Furthermore, the administration of anti-interleukin 6 monoclonal antibodies in murine models of experimental colitis prevented disease associated wasting and inhibited development of lesions (Ito, 2003); thus, the beneficial effect of probiotic administration seems to lie in the manipulation of the immunological profile.

However, in a study performed on healthy women, oral feeding of frozen concentrates of *L. fermentum* RC-14 and *L. rhamnosus* GR-1 (3mL, 2× daily for 14 days) there was no observed change in cytokine profiles (IL-6 and IFNγ) (Gardiner et al., 2002). Yet in critically ill patients, administration of ProViva, a probiotic preparation of *L. plantarum* 299v, there was a marked decrease in IL-6 (McNaught et al., 2005). Taken together, this suggests that probiotic administration did not modify immune function in healthy individual, however, in critically ill individuals with immune systems in an altered state, probiotic administration may prove beneficial by modulating pro-inflammatory cytokine profile.

More importantly, we found that fermented milk supernatants had significant inhibitory activity, decreasing IL-6 secretion back to the levels of untreated cells. The majority of the studies have used heat killed, viable or isolated cell wall components, as have we, however there are many inherent problems associated with using these treatments. The use of cell free fermented milk supernatants could circumvent the possible concerns of developing bacteremia (De Groote et al., 2005; Land et al., 2005; Salminen et al., 2002) or fever and malaise due to live bacterial treatments (Patard et al., 2003; Lockyer and Gillat, 2001). This could be of particular importance in the treatment of disorders where the immune system is compromised. From an industrial perspective, this circumvents the problem of viability of LAB in food products as a function of shelf life.

Although, we did not identify the active component in fermented milk that down regulated IL-6 secretion, we measured the effect of several individual milk proteins present in fermented milk (Table 7.8). As previously mentioned these proteins are liberated from milk via bacterial enzymatic or lactic acid digestion of the protein structure. The milk proteins can be divided into two categories based upon their origins, casein and whey protein fractions. It is interesting to note that both casein hydrolysate and sodium caseinate significantly inhibited IL-6 secretion; since in previous studies on TNF and NO stimulation, sodium caseinate had no effect. Conversely, other researchers have found that β-casein and αs1- casein have stimulatory activity on several normal and cancer cells lines of human origin (Kawahara et al., 2004; Otani et al., 2003; Sandre et al., 2001). The differences in IL-6 action found in the present study could be due to several reasons. Since we used hydrolyzed casein and sodium caseinate as opposed to the purified individual proteins (αs-1 and β-casein), perhaps the presence of both of the proteins triggered inhibitory activity in macrophage cells, whereas individual proteins may have stimulatory activity. However, we did find inhibitory activity for β-casomorphin-7, a derivative of β-casein (Schlimme and Meisel, 1995). The cells lines used in our experiment are also different than those used by Kawahara and colleagues (2004), this could account for the differential response as well. However, we can not definitively make this conclusion, further experimentation is required.

In addition, the whey protein fractions, β-lactoglobulin, α-lactalbumin and lactoferrin also displayed significant inhibitory activity. Endotoxins and lipopolysaccharides derived from gram-negative bacteria are present in the gut lumen. Binding of these components to macrophage cell surface membrane CD14, triggers activation of the inflammatory cascade releasing TNFα and IL-6 (Amati, 2003). Lactoferrin has high affinity for CD14 (Amati, 2003) and for LPS (Kruzel et al., 2002), which prevents or competitively inhibits binding of LPS/endotoxins to CD14. It is suggested that through this mechanism lactoferrin can be a potent inhibitor of IL-6 secretion.

It is well documented that Lf exerts antimicrobial activity through chelating iron and limiting its availability to gram-negative indigenous bacteria (Brock, 1980; Griffiths et a., 2003). Since an imbalance between beneficial and deleterious bacteria has been implicated as a causative factor in IBD development (Podolsky, 2002; Fiocchi, 1998), this could be a potential mechanism with which lactoferrin can alleviate inflammation, however, perhaps that potent inhibition of IL-6 secretion by lactoferrin, as demonstrated in the present experiment, is another mechanism. However, contrary to our results, *in vivo* administration of LF and *Bifidobacterium* did not inhibit IL-6 secretion (Griffiths, 2004); nor did administration of LF exert any IL-6 associated protective effect against LPS/*E. coli* induced endotoxemia in mice (Zimecki et al., 2004).

Interestingly, we found that α-lactalbumin and β-lactoglobulin have potent IL-6 inhibitory activity. A comprehensive search with several medical databases did not identify any previous studies that have found similar results. Taken together, all of these components, proteins/peptides derived from the fermentation process, cellular wall components and peptidoglycan contribute to down regulating the inflammatory reaction.

7.4. CONCLUSION

The immune system is a delicate balance act between pro and anti-inflammatory agents. The ability to manipulate this would be a powerful tool in the prevention/treatment of many diseases, thus the search for agents with immunomodulatory capacity is widespread. In this study we have demonstrated that several milk derived proteins and peptides and dairy LAB have moderate to potent immunomodulatory capacity, with respect to TNF α and IL-6. The development of a food-based product, specifically a dairy product, with immunomodulatory capacity for use in therapeutic applications and in prevention has many inherent advantages. Many pharmaceutical preparations are associated with a plethora of potential side effects and are usually quite expensive; while, dairy products are widely available, in many forms and well tolerated. In addition, the majority of the components, i.e. LAB and bioactive proteins/peptides, are naturally present or already commercially used for dairy production, thus there is no need to further isolate active components.

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Table 7.1. The Effect of Heat Killed LAB and Peptidoglycan on TNF α Production in Unstimulated RAW 264.7 Mouse Macrophage Cells[a].

Bacteria	Treatment	TNF ×10^2 (pg/mL)
Control	**Positive**	53.80±11.39
Control	**Negative**	1.64±0.06
***L. acidophilus* NRC 13019**	**Heat Killed**	16.36±9.67*
***L. acidophilus* NRC 13017**	**Heat Killed**	4.40±0.76
***L. casei* ADA 03**	**Heat Killed**	8.40±0.83
	PG 1000	4.03±0.80
	PG 800	11.29±0.88
	PG 600	8.66±8.2
***L. casei* NRC 13005**	**Heat Killed**	19.07±2.94*
***L. acidophilus* MR 100**	**Heat Killed**	25.93±2.85*
***L. bulgaricus* MR 110**	**Heat Killed**	18.28±5.83*
***L. casei* CSCC 2601**	**Heat Killed**	10.70±3.59
Yogurt Culture YC085	**Heat Killed**	6.61±1.21
Mixture	**Heat Killed**	6.41±0.62

a. In 96 well plate, RAW 264.7 mouse macrophages were seeded at a concentration of 1 × 10^6 cells/well and allowed to attach overnight. The cells were then washed with fresh DMEM to remove all non attached cells. Into each well, 100 μL of each treatment was added. *Salmonella typhimurium* LPS was used as a positive control. The plates were then incubated at 37°C (5% CO_2/95 % air) for 48 hours. After incubation, the plates were centrifuges and the supernatants were carefully collected for concentration of tumour necrosis factor. TNFα was measured using the BD OptEIA mouse TNF (Mono/Mono) ELISA kit from BD Biosciences Pharmingen (Missauga, ON, Canada).

* Significantly different than the negative control ($p \leq 0.05$); each experiment was performed a minimum of three times in triplicate. Treatments not shown (Live and fermented milk supernatants) did not stimulate any secretion of TNF above the negative control. ($p \leq 0.05$).

Table 7.2. The Effect of Milk Derived Protein/Peptides on TNF α Production in Unstimulated RAW 264.7 Mouse Macrophage Cells[a].

Milk Protein	Concentration	TNF α × 10^2 (pg/mL)
Control	**Positive**	53.80±11.39
Control	**Negative**	1.64±0.06
Casein Hydrolysate	**20 mg/mL**	5.03±0.43
	10 mg/mL	3.27±0.66
β-Casomorphin-7	**120 μg/mL**	4.57±0.38
	62.5 μg/mL	1.74±0.40
	31.25 μg/mL	1.15±0.16
Lactoferrin	**20 μg/mL**	3.16±0.56
α Lactalbumin	**35 μg/mL**	4.61±1.3
	25 μg/mL	5.46±2.2
	10 μg/mL	13.39±3.1*

a. In 96 well plate, RAW 264.7 mouse macrophages were seeded at a concentration of 1 × 10^6 cells/well and allowed to attach overnight. The cells were then washed with fresh DMEM to remove all non attached cells. Into each well, 100 μL of each treatment was added. *Salmonella typhimurium* LPS was used as a positive control. The plates were then incubated at 37°C (5% CO_2/95 % air) for 48 hours. After incubation, the plates were centrifuges and the supernatants were carefully collected for concentration of tumour necrosis factor. TNFα was measured using the BD OptEIA mouse TNF (Mono/Mono) ELISA kit from BD Biosciences Pharmingen (Missauga, ON, Canada).

* Significantly different than the negative control ($p \leq 0.05$); each experiment was performed a minimum of three times in triplicate.

Table 7.3. The Inhibitory Effect of Dairy Derived LAB on TNF α Production in *Salmonella typhimurium* LPS Stimulated RAW 264.7 Mouse Macrophage Cells.

LAB	Treatment	TNF α (pg/mL)
L. acidophilus **NRC 13019**	**Live**	30.26*
	24 Hour SN	14.71*
	48 Hour SN	35.48*
	HK	4569.60
L. acidophilus **NRC 13017**	**Live**	31.51*
	24 Hour SN	2.35*
	48 Hour SN	26.08*
	HK	4065.29
L. casei **ADA 03**	**Live**	33.50*
	24 Hour SN	38.42*
	48 Hour SN	44.02*
	PG 800	2713.07
	PG 600	2079.74
	HK	5727.85
L. casei **NRC 13005**	**Live**	26.58*
	24 Hour SN	29.95*
	48 Hour SN	37.25*
L. acidophilus **MR 100**	**Live**	29.64*
	24 Hour SN	17.101*
	48 Hour SN	38.95*
L. bulgaricus **MR 110**	**Live**	31.34*
	24 Hour SN	28.56*
	48 Hour SN	26.39*
L. casei **CSCC 2601**	**Live**	33.98*
	24 Hour SN	27.07*
	48 Hour SN	27.80*
Yogurt Culture YC085	**Live**	36.67*
	24 Hour SN	30.57*
	48 Hour SN	85.12*
Mixture	**Live**	59.20*
	24 Hour SN	36.29*
	48 Hour SN	39.07*

a. In 96 well plate, RAW 264.7 mouse macrophages were seeded at a concentration of 1 × 10^6 cells/well and allowed to attach overnight. The cells were then washed with fresh DMEM; 100 µL of LPS (in DMEM) was added to each well, except for the negative control. Into each well, 100 µL of each treatment was added. The plates were incubated at 37°C (5% CO_2/95 % air) for 48 hours. After incubation, the supernatants were carefully collected. TNFα was measured using the BD OptEIA mouse TNF (Mono/Mono) ELISA kit. * Significantly different than the positive control ($p \leq 0.05$); each experiment was performed a minimum of three times in triplicate. Positive Control: 5380.2 pg/mL; Negative Control: 246.03 pg/mL;

Table 7.4. The Stimulatory Effect of Dairy Derived LAB on TNF α Production in *Salmonella typhimurium* LPS Stimulated RAW 264.7 Mouse Macrophage Cells.

LAB	Treatment	TNF α (pg/mL)	% Augmentation
Control	**Positive**	5380.20±1139	-
Control	**Negative**	246.03±13	-
***L. casei* ADA 03**	**PG 1000**	12067.37±2964*	124
***L. casei* NRC 13005**	**Heat Killed**	14789.46±2751*	175
***L. acidophilus* MR 100**	**Heat Killed**	21595.18±5154*	301
***L. bulgaricus* MR 110**	**Heat Killed**	11972.33±2391*	123
***L. casei* CSCC 2601**	**Heat Killed**	18713.07±3516*	248
Yogurt Culture YC085	**Heat Killed**	21198.49±3304*	294
Mixture	**Heat Killed**	7672.79±1508	43

a. In 96 well plate, RAW 264.7 mouse macrophages were seeded at a concentration of 1×10^6 cells/well and allowed to attach overnight. The cells were then washed with fresh DMEM; 100 µL of LPS (in DMEM) was added to each well, except for the negative control. Into each well, 100 µL of each treatment was added. The plates were incubated at 37°C (5% CO_2/95 % air) for 48 hours. After incubation, the supernatants were carefully collected. TNFα was measured using the BD OptEIA mouse TNF (Mono/Mono) ELISA kit.

* Significantly different than the positive control ($p \leq 0.05$); each experiment was performed a minimum of three times in triplicate.

Table 7.5. The Effect of Dairy Derived Milk Proteins on TNF α Production in *Salmonella typhimurium* LPS Stimulated RAW 264.7 Mouse Macrophage Cells

Milk Protein	Concentration	TNF α (pg/mL)
Control	**Positive**	5380.20
Control	**Negative**	246.03
Casein Hydrolysate	**40 mg/mL**	47.65*
	20 mg/mL	1450.92
	10 mg/mL	10137.14$^{\Psi}$
Sodium Caseinate	**75 mg/mL**	172.50*
	37.5 mg/mL	151.77*
	18.75 mg/mL	144.00*
β-lactoglobulin	**120 mg/mL**	28.09*
	90 mg/mL	97.58*
	60 mg/mL	258.62*
	30 mg/mL	62.61*
	15 mg/mL	150.07*
β-casomorphin-7	**125 μg/mL**	988.25*
	62.5 μg/mL	2309.74
	31.25 μg/mL	2201.22
Lactoferrin	**500 μg/mL**	333.32*
	200 μg/mL	385*
	100 μg/mL	340.85*
	20 μg/mL	928.96*
α-lactalbumin	**35 μg/mL**	4270.48
	25 μg/mL	2329.28
	10 μg/mL	1961.47

a. In 96 well plate, RAW 264.7 mouse macrophages were seeded at a concentration of 1×10^6 cells/well and allowed to attach overnight. The cells were then washed with fresh DMEM; 100 μL of LPS (in DMEM) was added to each well, except for the negative control. Into each well, 100 μL of each treatment was added. The plates were incubated at 37°C (5% CO_2/95 % air) for 48 hours. After incubation, the supernatants were carefully collected. TNFα was measured using the BD OptEIA mouse TNF (Mono/Mono) ELISA kit. * Inhibitory Activity: Significantly different than positive control ($p \leq 0.05$); $^{\Psi}$ Augmentation Activity: significantly different than positive control ($p \leq 0.05$)'

Table 7.6. The Effect of Dairy Derived Milk Proteins and LAB on IL 6 Production in Unstimulated RAW 264.7 Mouse Macrophage Cells.

Probiotic/Milk Protein	Treatment/Concentration$^{\Psi}$	IL 6 × 10^2 (pg/mL)
L. acidophilus NRC 13019	Heat Killed	11.62±0.29*
L. acidophilus NRC 13017	Heat Killed	2.46±.0.11
L. casei ADA 03	Heat Killed	7.93±0.07*
	PG 1000	14.00±0.34*
	PG 800	22.29±0.99*
	PG 600	24.71±0.33*
L. casei NRC 13005	Heat Killed	15.65±0.30*
L. acidophilus MR 100	Heat Killed	19.41±0.40*
L. bulgaricus MR 110	Heat Killed	4.05±0.14*
L. casei CSCC 2601	Heat Killed	2.38±.05
Yogurt Culture YC085	Heat Killed	15.25±0.30*
Mixture	Heat Killed	13.48±0.38*
β-Lactoglobulin	60 mg/mL	0.89±.06
	30 mg/mL	2.66±0.22
Lactoferrin	20 μg/mL	1.77±0.17

a. In 96 well plate, RAW 264.7 mouse macrophages were seeded at a concentration of 1 × 10^6 cells/well and allowed to attach overnight. The cells were then washed with fresh DMEM to remove all non attached cells. Into each well, 100 μL of each treatment was added. *Salmonella typhimurium* LPS was used as a positive control. The plates were then incubated at 37°C (5% CO_2/95 % air) for 48 hours. After incubation, the plates were centrifuged and the supernatants were carefully collected. IL 6 was measured using the BD OptEIA mouse IL 6 ELISA kit from BD Biosciences Pharmingen (Missauga, ON, Canada).

* Significantly different than the negative control ($p \leq 0.05$); each experiment was performed a minimum three times in triplicate. $^{\Psi}$ Samples tested but not within this table did not stimulate secretion of IL-6 above the negative control

Table 7.7. The Inhibitory Effect of Dairy Derived LAB on IL 6 Production in *Salmonella typhimurium* LPS Stimulated RAW 264.7 Mouse Macrophage Cells[ab].

LAB	Treatment	IL 6 × 10^2 pg/mL	% Inhibition[¥]
***L. acidophilus* NRC 13019**	**Heat Killed**	25.26±5.02	88.61*
***L. acidophilus* NRC 13017**	**Heat Killed**	11.33±2.16	94.99*
***L. casei* ADA 03**	**Heat Killed**	22.29±1.98	90.43*
	PG 1000	54.11±8.76	75.41*
	PG 800	10.29±1.30	95.46*
	PG 600	4.11±1.96	98.29*
***L. casei* NRC 13005**	**Heat Killed**	27.10±1.56	87.77*
***L. acidophilus* MR 100**	**Heat Killed**	25.08±2.55	88.69*
***L. bulgaricus* MR 110**	**Heat Killed**	26.05±.56	88.25*
***L. casei* CSCC 2601**	**Heat Killed**	14.59±1.41	93.49*
Yogurt Culture YC 085	**Heat Killed**	13.51±0.94	93.99*
Mixture	**Heat Killed**	25.01±5.01	88.72*

[¥] LPS positive control: 218.53×10^2; % Inhibition: 100-[(Treatment/Positive Control)*100]

a. In 96 well plate, RAW 264.7 mouse macrophages were seeded at a concentration of 1×10^6 cells/well and allowed to attach overnight. The cells were then washed with fresh DMEM; 100 μL of LPS (in DMEM) was added to each well, except for the negative control. Into each well, 100 μL of each treatment was added. The plates were incubated at 37°C (5% CO_2/95 % air) for 48 hours. After incubation, the supernatants were carefully collected. IL 6 was measured using the BD OptEIA mouse IL 6 ELISA kit.

b. All of the live and supernatant fractions (24 and 48 hour) tested, significantly ($p \le 0.05$) inhibited of IL 6 secretion by 100%

* Significantly different than the positive control ($p \le 0.05$); each experiment was performed a minimum of three times in triplicate.

Table 7.8. The Inhibitory Effect of Dairy Derived Milk Proteins on IL 6 Production in *Salmonella typhimurium* LPS stimulated RAW 264.7 Mouse Macrophage Cells[a].

Milk Protein	**Concentration**	**IL 6 × 10^2 (pg/mL)[¥]**	**% Inhibition**
Casein Hydrolysate	**40 mg/mL**	5.02±0.27	97.87*
	20 mg/mL	5.11±1.27	97.83*
	10 mg/mL	3.32±0.50	98.65*
Sodium Caseinate	**75 mg/mL**	1.91±0.58	99.33*
	37.5 mg/mL	2.74±0.45	98.96*
	18.75 mg/mL	3.40±0.37	98.62*
β-Lactoglobulin	**120 mg/mL**	1.82±0.39	99.34*
	90 mg/mL	13.27±0.24	94.10*
	60 mg/mL	1.37±0.11	99.54*
	30 mg/mL	19.39±1.89	91.30*
	15 mg/mL	3.73±0.38	98.46*
β-Casomorphin-7	**125 µg/mL**	13.81±3.04	93.85*
	62.5 µg/mL	11.69±3.35	94.82*
	31.25 µg/mL	30.73±39.06	85.96*
Lactoferrin	**500 µg/mL**	13.94±0.56	93.79*
	200 µg/mL	13.80±2.13	93.85*
	100 µg/mL	14.84±2.58	93.38*
	20 µg/mL	18.24±0.70	91.82*
α-Lactalbumin	**35 µg/mL**	15.38±1.76	93.13*
	25 µg/mL	13.15±1.16	94.38*
	10 µg/mL	14.89±1.71	93.36*

a. In 96 well plate, RAW 264.7 mouse macrophages were seeded at a concentration of 1×10^6 cells/well and allowed to attach overnight. The cells were then washed with fresh DMEM; 100 µL of LPS (in DMEM) was added to each well, except for the negative control. Into each well, 100 µL of each treatment was added. The plates were incubated at 37°C (5% CO_2/95 % air) for 48 hours. After incubation, the supernatants were carefully collected. IL 6 was measured using the BD OptEIA mouse IL 6 ELISA kit.

[¥] LPS positive control: 218.53×10^2;% Inhibition: 100-[(Treatment/Positive Control)*100]

* Significantly different than the positive control ($p \leq 0.05$); each experiment was performed a minimum of three times in triplicate.

CHAPTER 8

Final Remarks

8.1 INTRODUCTION

The landscape of the food industry has changed dramatically with the preferences of the consumer. In the 80's, there was a dramatic increase in products that were low in fat and highly unpalatable. Whereas in the 90's, the consumer became more conscious of not only low fat foods, but food items that were highly palatable, convenient and healthful beyond basic nutrition. The concept of functional foods, food that has beneficial effects beyond nutrition, has begun to become a key aspect of consumer choice and demand. Thus the food industry has embarked on developing and producing these products to meet consumer demands.

Functional foods are an ever expanding market, particularly in Australia, Europe, Japan and North America. The term functional foods first originated in Japan in the 1980's. Japan is the only country with a legal definition for functional foods, however, other countries have developed working definitions. According the Health Canada, the working definition for functional foods states that a functional food is similar in appearance to, or may be a conventional food, is consumed as part of a usual diet and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions. In 1996, the functional food market was estimated to be worth $6.6 billion US (Hilliam, 1998), it is currently estimated to be worth between $10-30 billion US, depending on the definition of functional foods used (Agriculture and Agri-food Canada, 2001). However, due to the uncertain and restrictive regulatory environment currently in Canada many companies are dissuaded from pursuing the sale of such products in Canada. As a result, the functional food market is deficient within Canadian markets as opposed to other countries like the United States, Japan and Europe.

However, according to Agriculture and Agri-food Canada (2001), there is tremendous potential in developing functional foods from dairy. The Food and Drugs

Act and Regulations has not placed any restrictions upon the introduction of probiotic yogurts or fermented milk drinks into the market, except for products making specific health claims. Worldwide dairy food sales were worth an estimated $70 billion US in 2004, while it was estimated to be worth $4.6 billion (Canadian) in Canada alone. In Canada, both consumption and production have dramatically increased in the last two decades. In 1980, consumption of yogurt was 1.61L/per capita, whereas in 2004, consumption had increased almost 400% to 6.28 L/per capita. Yogurt production in 1990 was just above 80 000 000 Kg/year, whereas now it is well over 200 000 000 kg/year (Agriculture and Agri-food Canada, 2005). This illustrates the trend for increasing consumption of and demand for healthful dairy products. Recently new products, such as Activia™ and Astro Yogurt™, have been introduced into the dairy food market advertising the presence of live probiotic cultures.

However, unfortunately, with increasing consumer demand for more functional food products there are also many products being introduced into the market place with unsubstantiated health claims. This underscores the fundamental need to not only develop more products to meet consumer demand but to develop products that have rigorous scientific data supporting the functional food claims.

Dairy product production represents the fourth largest sector of food production in Canada (Agriculture and Agri-food Canada, 2005). Due to the presence of various lactic acid bacteria (LAB) used to ferment milk and the consequent liberation of biogenics from the native structure of milk, dairy products represents significant potential as functional foods or source of functional food ingredients. Even though much research has been performed on the efficacy of probiotic LAB and biogenics in the modulation of many disorders, there are still many questions that remain to be answered. Although, clinical trials and animal studies have demonstrated efficacy, there is still a strong lack of understanding of the fundamental mechanism through which probiotic LAB and biogenics exert their effects. Furthermore, the majority of the current LAB being used are human isolates, dairy derived LAB represent a significant and thus far only minorly exploited opportunity for discovering other sources of LAB with probiotic potential.

8.2 SUMMARY OF RESEARCH FINDINGS

The overall objective of this research was to:

- Identify new functional ingredients derived from dairy LAB and proteins that possess bioactive properties that can be used as dietary adjuvants in the treatment or prevention of gastrointestinal disorders.

The following aspects of health benefits of LAB and biogenics were studied:

- Antimutagenicity
- Adhesion properties
- Cytotoxicity
- Apoptosis and necrosis
- Nitric Oxide
- Immunomodulatory effects (TNFα and IL-6)
- Biological properties of selected proteins and peptides.

Conducted research program resulted in the following general conclusions.

Antimutagenic Studies

L. casei ADA 03 (Live culture, 24 hour 7 day fermented milk samples, 24 hour fermented milk supernatant and peptidoglycan) and several milk proteins (sodium caseinate, β-lactoglobulin, casein hydrolysate and β-casomorphin-7) were tested for their ability to inhibit 2-aminofluorene (2AF) induced mutation of *Salmonella typhimurium* TA98. We have demonstrated that *Lactobacillus casei* ADA 03 (live and fermented milk samples) has significant antimutagenic activity against 2 AF induced mutation of *Salmonella typhimurium* TA98. In addition, we have shown that bioactive peptides, such as β-casomorphin-7, and β-lactoglobulin, that are released from milk during fermentation also have antimutagenic activity. Mutations to DNA may ultimately result in the development of cancer. The ability to inhibit mutation or to augment the body's DNA repair system by a dairy product has a significant economic impact upon dairy industry and therapeutic and economic impact upon the health care system. Further research is required to fully characterize the health benefits of dairy derived LAB and protein/peptides; with the ultimate goal being the development of a dairy based product that can act as a dietary immunoadjuvant to cancer treatment.

Adhesion Studies

Dairy isolated LAB (*Lactobacillus acidophilus* MR 100, *Lactobacillus bulgaricus* MR 110, *Lactobacillus acidophilus* NRC 13017, *Lactobacillus acidophilus* NRC 13019, *Lactobacillus casei* ADA 03, *Lactobacillus casei* ADA 05, Yogurt Culture YC085 and *Lactobacillus casei* subsp. *casei* CSCC 2601) were tested for their adhesive properties to HT29 colon cancer cell line. Furthermore, we also applied chemical and physical treatments to try to determine components involved in adherence. We found that adhesion occurred in a strain dependent manner to HT29 cells; however, all strains were highly adhesive. LAB also adhered to HT29 through divalent cations interactions between intestinal epithelial cell surface and bacterial cells, carbohydrate and protein moieties and glycoprotein adherence mechanisms. The type of adherence mechanisms exhibited was strain dependent.

Nitric Oxide Studies

Dairy isolated LAB (*Lactobacillus acidophilus* MR 100, *Lactobacillus bulgaricus* MR 110, *Lactobacillus acidophilus* NRC 13017, *Lactobacillus acidophilus* NRC 13019, *Lactobacillus casei* ADA 03, *Lactobacillus casei* ADA 05, Yogurt Culture YC085 and *Lactobacillus casei* subsp. *casei* CSCC 2601) and proteins/peptides (Sodium caseinate, β-lactoglobulin, lactoferrin, α-lactalbumin, casein hydrolysate and β-casomorphin-7) were tested for their ability to modulate nitric oxide secretion from naive or activated RAW 264.7 mouse macrophage cell line. We found that NO secretion was stimulated by heat killed samples of LAB and several milk proteins from naive RAW 264.7 mouse macrophage cells. In activated mouse macrophage cells dairy derived LAB live and fermented milk supernatants and several milk proteins were potent inhibitors of NO secretion. Interestingly, several of the milk proteins were able to augment NO secretion from activated macrophage cells. NO oxide is a potent modulator of immune response both in colon cancer and inflammatory bowel disease. We have shown that various species/components of LAB and milk proteins can possibly be used as dietary adjuvants in the treatment/prevention of these diseases through modulation of NO secretion from macrophage cells; however, it should be cautioned that the immunological picture is not as simplistic. A large problem with using immunomodulating substances in the treatment of cancer and inflammation is that many of the cytokines and cell signaling mediators play paradoxical roles in disease progression and resolution; depending upon factors such as concentration of the mediator, stage of the disease, location of the disease and many other complex factors NO can have a beneficial or deleterious effect. Thus before dairy derived LAB and milk proteins can be ascribed as chemotherapeutic or IBD dietary adjuvants for treatment or prevention further extensive research needs to be performed.

Cytotoxicity Studies

Dairy isolated LAB (*Lactobacillus acidophilus* MR 100, *Lactobacillus bulgaricus* MR 110, *Lactobacillus acidophilus* NRC 13017, *Lactobacillus acidophilus* NRC 13019, *Lactobacillus casei* ADA 03, *Lactobacillus casei* ADA 05, Yogurt Culture YC 085and *Lactobacillus casei* subsp. *casei* CSCC 2601) and proteins/peptides (Sodium caseinate, β-lactoglobulin, lactoferrin, α-lactalbumin, casein hydrolysate and β-casomorphin-7) were tested for cytotoxic activity against colon cancer cell line HT29. We have demonstrated that several strains of LAB (live, heat killed and peptidoglycan), fermented milk supernatants and milk proteins/peptides have cytotoxic activity against colon cancer cell line HT 29. Studies demonstrate the immune modulatory activity of LAB in cancer, perhaps in addition to these mechanisms, LAB exhibit anticancer properties through direct cytotoxic action against cancer cells.

Apoptosis and Necrosis Studies

Dairy isolated LAB (*Lactobacillus acidophilus* MR 100, *Lactobacillus bulgaricus* MR 110, *Lactobacillus acidophilus* NRC 13017, *Lactobacillus acidophilus* NRC 13019, *Lactobacillus casei* ADA 03, *Lactobacillus casei* ADA 05, Yogurt Culture YC 085 and

Lactobacillus casei subsp. *casei* CSCC 2601) and proteins/peptides (Sodium caseinate, β-lactoglobulin, lactoferrin, α-lactalbumin, casein hydrolysate and β-casomorphin-7) were tested for apoptotic and necrotic activity against HT29 colon cancer cells. This study shows that dairy derived LAB and proteins can decrease viability in cancer cells through activation of apoptosis and necrosis. Apoptotic mechanisms are often defective in cancer cells, thus exogenous elements that can initiate apoptotic activity in cancer cells would be of therapeutic relevance. Furthermore, studies in the treatment of bladder cancer using *Bacillus Calmette Guerin* demonstrate mechanism of action is through induction of necrotic cell death. Unfortunately, there are many side effects associated with the use of BCG. Thus, development of other probiotic LAB treatment modalities that are associated with tolerability and low risk of infection is ideal. However, this study was only performed *in vitro* using immortalized cancer cell lines, larger animal and clinical studies need to be performed because we can definitively attribute anticancer attributes to these compound.

Immunomodulation Studies

The immune system is a delicate balance act between pro and anti-inflammatory agents. The ability to manipulate this would be a powerful tool in the treatment of many diseases, thus the search for agents with immunomodulatory capacity is widespread. Dairy isolated LAB (*Lactobacillus acidophilus* MR 100, *Lactobacillus bulgaricus* MR 110, *Lactobacillus acidophilus* NRC 13017, *Lactobacillus acidophilus* NRC 13019, *Lactobacillus casei* ADA 03, *Lactobacillus casei* ADA 05, Yogurt Culture YC 085 and *Lactobacillus casei* subsp. *casei* CSCC 2601) and proteins/peptides (Sodium caseinate, β-lactoglobulin, lactoferrin, α-lactalbumin, casein hydrolysate and β-casomorphin-7) were tested for immune modulatory activity. In particular we examine the effects of these treatments to stimulate or inhibit the production of inflammatory cytokines tumour necrosis factor and interleukin 6. In this study we have demonstrated that several milk derived proteins and peptides and dairy LAB have moderate to potent immunomodulatory capacity through stimulation or inhibition of TNF α and IL-6 secretion from activated and naive mouse macrophages. The development of a food-based product, specifically a dairy product, with immunomodulatory capacity for use in therapeutic applications and in prevention has many inherent advantages. Many pharmaceutical preparations are associated with a plethora of potential side effects and are usually quite expensive; while, dairy products are widely available, in many forms and well tolerated. In addition, the majority of the components, i.e. LAB and bioactive proteins/peptides, are naturally present or already commercially used for dairy production, thus it is not necessary to alter production processes to further isolate active components.

8.3 RECOMMENDATION FOR FUTURE RESEARCH

In all mammals, milk represents the first source of nutrition. Humans are unique from other mammals because they consume milk throughout the lifespan. Traditionally milk was consumed simply as a highly nutritious source of vitamins, minerals and proteins. However, recently focus has shifted to the bioactive properties of milk and dairy products. During production of dairy products, many bioactive ingredients are liberated from milk or LAB are added to milk. Specifically, interest in the LAB used to ferment milk and the bioactive proteins and peptides that are liberated from the native structure of milk as potential functional ingredients, is growing.

Particularly due to the ease with which probiotic yogurts or fermented milks can be introduced into the Canadian food marketplace, characterizing the bioactive properties of dairy derived LAB and proteins/peptides are essential. The results from the experiments presented in this thesis demonstrate the ability of dairy derived LAB and milk proteins to modulate various processes within the cell. However, further research is warranted to confirm and expand the findings presented in this thesis. Further research needs to be done to elucidate exact dosages, time of administration or type of functional ingredient administered. LAB and milk proteins associated food can be developed into specialized functional foods that could be used as dietary adjuvants to treatment or prevention. Understanding the precise areas in which these functional ingredients are effective would allow for the development of a cocktail of ingredients that would have modulatory effect on specific disease states. Currently, we have only related the findings of this thesis to colon cancer and inflammatory bowel disease. These diseases directly involve the lining of the gastrointestinal tract, thus represent an ideal target for functional dairy derived ingredients to act.

Indeed, we have found that certain strains of dairy derived LAB and biogenics could possibly be used as dietary adjuvants in the modulation of colon cancer due to their ability to survive transit through and adhere to the gastrointestinal tract, and consequently induce direct cytotoxic, apoptotic, necrotic cell death, modulate inflammatory mediators such as nitric oxide, tumour necrosis factor and interleukin 6 and inhibit mutations. These bioactivities could prove to have significant therapeutic potential. However further *in vitro* and *in vivo* studies need to be performed to further characterize their biological activities with in the body.

Furthermore, these same treatments could also aid in the modulation of intestinal inflammation in inflammatory bowel disease. Inflammatory bowel disease is characterized by a modified microflora and up regulation of inflammatory cytokines. Research is only beginning to suggest a strong role of excess interleukin 6 secretion in the etiology of IBD. Thus isolation of bioactive agents that have the ability to down regulate this cytokine would be of tremendous therapeutic value. We have shown that several strains of dairy derived LAB and proteins/peptides have strong inhibitory activity against IL-6 secretion from activated macrophage cells. *In vivo* studies using normal and disease specific animals, where these markers are measured after ingestion, are required to confirm this inhibitory activity.

Furthermore, the use of elemental diets composed primarily of casein or whey protein, have been shown to be successful in the treatment of IBD. However, very few studies have tried to elucidate the precise components of these elemental diets that exert the beneficial effects nor the mechanism of action. As we have observed in this present study, all the individual proteins tested exert significant inhibitory activity upon the secretion of inflammatory mediators NO, TNFα and IL-6. Administration of these proteins to animal models of colitis and the measurement of colitic activity, NO, IL-6 and

TNFα concentrations within the blood could shed light on the mechanism through which elemental diets composed of whey and casein are able to control colitis symptoms.

Although we have demonstrated *in vitro* efficacy of dairy derived LAB and milk proteins in modulating NO, TNF-α and IL-6 secretion in mouse macrophages, administration of dairy derived LAB and milk proteins in *in vivo* models needs to be elucidated. The administration of these to normal animals and genetically modified or disease specific animals to determine changes in the inflammatory mediators would confirm or refute the current results that we have obtained through *in vitro* measurements

Furthermore, in the present research we have focused upon the ability of dairy derived LAB and milk proteins to alter Th1 or pro-inflammatory responses. However, there is a delicate balance between inflammatory (Th1) and anti-inflammatory (Th2) responses within the body; an imbalance towards either direction could results in disease development. Thus, subsequent studies should also focus upon the effect of these treatments upon the Th2 or anti-inflammatory responses using both *in vitro* and *in vivo* studies.

We have demonstrated that *L. casei* ADA03 and several milk derived proteins/peptides have antimutagenic activities against chemical carcinogen, 2-aminofluorene. However, further research needs to be done to elucidate how this antimutagenic activity occurred. As previously discussed damage to DNA is an initial step in cancer development. Determining if these dairy derived LAB and milk proteins/peptides exert antimutagenic activity through directly preventing DNA damage or physically binding the carcinogen, thus preventing carcinogen interaction with the intestinal epithelial surface, would provide further in sight into the mechanistic actions of LAB.

We have also identified that proteinaceous, carbohydrate and metallic ion adhesion factors are involved in the adherence of these strains to colon cancer cells. However, we have not identified if these factors are directly involved or required for initiation of bioactivities. Applying similar chemical/physical treatments to bacteria prior immune stimulation, cellular death assays and consequent animal studies, would provide evidence into the mechanisms required for immune modulation and cellular death induction.

Furthermore, in this current thesis we have attempted to develop a cocktail of dairy derived bacterial strains. The cocktail of bacterial strains exerted a variety of biological activities; however future research should involve concentrating the bacteria cocktail to increase the total bacterial load. Moreover, not only have we identified bacterial strains with biological activities, we have also identified milk proteins/peptides with strong bioactive properties. Further research is required to develop additional cocktails and fermented milk products that contain selected LAB and milk proteins, based upon their range of bioactivities and the target disease state.

The ultimate aim of this research project is to identify dairy derived LAB and protein/peptides that have potential as functional ingredients. As previously discussed due to the restricting regulatory atmosphere present in Canada, many products developed for functional bioactive characteristics have not been made available within Canada. However, probiotic yogurts and fermented drinks are not restricted under the same legislation and thus provide a significant market with which to introduce food products with functional ingredients. Thus the development of a milk product fermented with LAB selected specifically for its range of bioactivities could be used as dietary adjuvants in prevention and treatment of many diseases. Development of such products should also be a component of further research.

The functional foods market in Canada is severely lagging in comparison to European, Japanese and Australian markets. Restrictive legislation against the sale of products without sound scientific research is inhibitory towards the marketing of many products, however, is ultimately employed to protect the consumer. Fortunately within Canada, this legislation does not apply to probiotic yogurts and fermented milk drinks unless a specific health claim is to be made. This provides an extremely significant opportunity for the dairy industry to develop products from LAB and associated milk proteins that have functional characteristics without having to deal with regulatory inhibitions. However, to maintain consumer and scientific legitimacy, these products need to be well characterized and thoroughly researched with rigorous scientific methods.

8.4 REFERENCES

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